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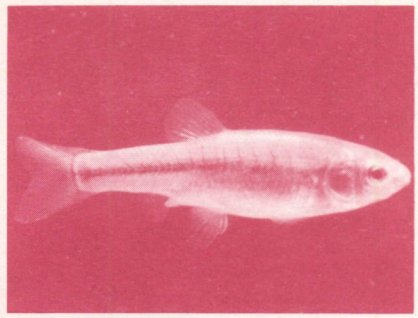

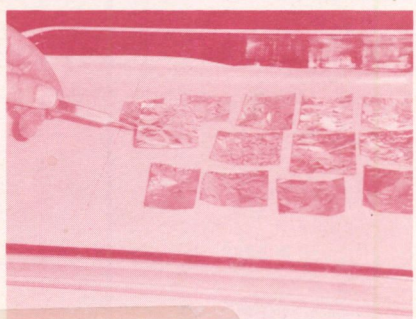

Biological Test Method:

Test of Larval Growth and Survival Using Fathead Minnows

Report EPS 1/RM/22
February 1992

Canada

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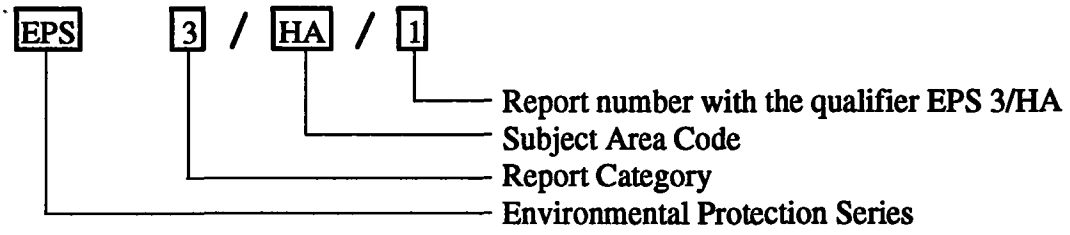

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Abstract

*Methods recommended by Environment Canada for performing toxicity tests of seven days' duration, that measure growth and survival of very young (larval) fathead minnows (*Pimephales promelas*), are described in this report. Because such early life-stages are usually a sensitive part of the life cycle of a fish, the test should be considered as a powerful and meaningful sublethal assay. The test may be run with five concentrations of test material to determine the threshold of effect, or with one concentration as a regulatory or pass/fail test.*

Procedures are given for culturing fathead minnows in the laboratory, obtaining eggs, and hatching the young for use in the tests. General or universal conditions and procedures are outlined for testing a variety of materials for their effects on larval growth and mortality. Additional specific conditions and procedures are stipulated for testing samples of chemicals, effluents, elutriates, leachates, or receiving waters. Instructions and requirements are included on test facilities, handling and storing samples, preparing test solutions and initiating tests, specific test conditions, appropriate observations and measurements, endpoints and methods of calculation, and the use of reference toxicants.

Résumé

Le présent document expose les méthodes recommandées par Environnement Canada pour l'exécution d'essais de toxicité d'une durée de sept jours qui permettent de mesurer la croissance et la survie de têtes-de-boule (Pimephales promelas) *au stade larvaire. Étant donné que ce premier stade constitue normalement une partie sensible du cycle biologique d'un poisson, on devrait considérer cet essai comme un essai sublétal puissant et significatif. On peut procéder à l'essai avec cinq concentrations de la substance à expérimenter afin de déterminer le seuil d'effet, ou avec une seule concentration à titre d'essai réglementaire ou d'essai à résultat unique.*

Ce document présente des méthodes pour l'élevage des têtes-de-boule en laboratoire, l'obtention d'oeufs et l'éclosion de larves devant servir aux essais. Il présente les conditions et méthodes générales ou universelles permettant de réaliser des essais sur un large éventail de substances pour déterminer leur effet sur la croissance et sur la mortalité des larves. On y précise aussi d'autres conditions et méthodes propres à l'évaluation d'échantillons de produits chimiques, d'effluents, d'élutriats, de lixiviats ou de milieux récepteurs. Le lecteur y trouvera des instructions et des exigences concernant les installations d'essai, la manipulation et le stockage des échantillons, la préparation des solutions d'essai et la mise en route des essais, les conditions prescrites pour les essais, les observations et mesures appropriées, les résultats des essais, les méthodes de calcul et l'utilisation de produits toxiques de référence.

Foreword

This is one of a series of ***recommended methods*** *for measuring and assessing the aquatic biological effects of toxic materials. Recommended methods are those which have been evaluated by Conservation and Protection (C&P), and are favoured:*

- *for use in C&P aquatic toxicity laboratories;*
- *for testing which is contracted out by Environment Canada or requested from outside agencies or industry;*
- *in the absence of more specific instructions, such as are contained in regulations; and*
- *as a foundation for the provision of very explicit instructions as might be required in a regulatory program or standard reference method.*

The different types of tests included in this series were selected on the basis of their acceptability for the needs of environmental protection and management programs carried out by Environment Canada. These documents are intended to provide guidance and to facilitate the use of consistent, appropriate, and comprehensive procedures for obtaining data on toxic effects of samples of chemical, effluent, elutriate, leachate, receiving water, or, where appropriate, sediment.

Mention of trade names in this document does not constitute endorsement by Environment Canada; other products with similar value are available.

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List of Abbreviations and Chemical Formulae

°C . . . . . .degree(s) Celsius

$CaCO_3$ . . . . . .calcium carbonate

cm . . . . . .centimetre(s)

d . . . . . .day(s)

DO . . . . . .dissolved oxygen (concentration)

g . . . . . .gram(s)

g/kg . . . . . .grams per kilogram

h . . . . . .hour(s)

HCl . . . . . .hydrochloric acid

H_2O . . . . . .water

IC_p . . . . . .inhibiting concentration for a (specified) percentage effect

L . . . . . .litre(s)

LC . . . . . .lethal concentration

LC_{50} . . . . . .median lethal concentration

LOEC . . . . . .lowest-observed-effect concentration

LT_{50} . . . . . .time to 50% mortality (lethality)

m . . . . . .metre(s)

mg . . . . . .milligram(s)

min . . . . . .minute(s)

mL . . . . . .millilitre(s)

mm . . . . . .millimetre(s)

mS . . . . . .millisiemen(s)

N . . . . . .Normal

NaOH . . . . . .sodium hydroxide

NOEC . . . . . .no-observed-effect concentration

P . . . . . .probability

SD . . . . . .standard deviation

SI . . . . . .Système international d'unités

sp. . . . . .species

TEC . . . . . .threshold-effect concentration

TIE . . . . . .toxicity identification evaluation

TM (™) . . . . . .Trade Mark

µg . . . . . .microgram(s)

µm . . . . . .micrometre(s)

> . . . . . .greater than

< . . . . . .less than

≥ . . . . . .greater than or equal to

≤ . . . . . .less than or equal to

± . . . . . .plus or minus

% . . . . . .percentage

‰ . . . . . .parts per thousand

Terminology

Note: all definitions are given in the context of the procedures in this report, and might not be appropriate in another context.

Grammatical Terms

Must is used to express an absolute requirement.

Should is used to state that the specified condition or procedure is recommended and ought to be met if possible.

May is used to mean "is (are) allowed to".

Can is used to mean "is (are) able to".

Might is used to mean "could".

General Technical Terms

Acclimation means to become physiologically adjusted to a particular level of one or more environmental variables such as temperature. The term usually refers to controlled laboratory conditions.

Compliance means in accordance with governmental permitting or regulatory requirements.

Conductivity is a numerical expression of the ability of an aqueous solution to carry an electric current. This ability depends on the concentrations of ions in solution, their valence and mobility, and on the solution's temperature. Conductivity is normally reported in the SI unit of millisiemens/metre, or as micromhos/centimetre (1 mS/m = 10 μmhos/cm).

Culture, as a noun, means the stock of animals or plants that is raised under defined and controlled conditions in order to produce healthy test organisms. As a verb, it means to carry out this procedure of raising organisms.

Dispersant is a chemical substance which reduces the surface tension between water and a hydrophobic substance (e.g., oil), thereby facilitating the dispersal of the hydrophobic material throughout the water as an emulsion.

Emulsifier is a chemical substance that aids the fine mixing (in the form of small droplets) within water, of an otherwise hydrophobic material.

Embryo means the undeveloped young fish, before it hatches from the egg. In literature on fathead minnows, the term 'embryo' is usually used instead of 'egg'.

Flocculation is the formation of a light, loose precipitate (i.e., a floc) from a solution.

Growth means increase in size or weight as the result of proliferation of new tissues. In this test it is limited to increase in dry weight.

Hardness is the concentration of cations in water that will react with a sodium soap to precipitate an insoluble residue. In general, hardness is a measure of the concentration of calcium and magnesium ions in water, and is expressed as mg/L calcium carbonate or equivalent.

Larva means a recently hatched fish which has physical characteristics other than those seen in the adult fish. The larval period begins with hatching of the embryo and lasts until the disappearance of the last vestige of the median fin fold and the appearance of a full complement of fin rays and spines. Fathead minnows are considered larvae for the first few days after hatching.

Lux is a unit of illumination based on units per square metre. One lux = 0.0929 foot-candles and one foot-candle = 10.76 lux.

Minimum Significant Difference (MSD) means the difference between groups (in this test with fathead minnows, the difference in average weights or average mortality) that would have to exist before it could be concluded that there was a significant difference between the groups. MSD is provided by Dunnett's multiple-range test, a standard statistical procedure.

Monitoring is the routine (e.g., daily, weekly, monthly, quarterly) checking of quality, or collection and reporting of information. In the context of this report, it means either the periodic (routine) checking and measurement of certain biological or water-quality variables, or the collection and testing of samples of-effluent, elutriate, leachate, or receiving water for toxicity.

Nauplius (plural nauplii) is the earliest larval stage characteristic of many marine crustaceans and some other invertebrates. It is microscopic, free-swimming, has only three pairs of appendages, and one median eye in the front of the head.

Percentage (%) is a concentration expressed in parts per hundred parts. One percentage represents one unit or part of material (e.g., effluent, elutriate, leachate, or receiving water) diluted with water to a total of 100 parts. Concentrations can be prepared on a volume-to-volume or weight-to-weight basis, and are expressed as the percentage of test material in the final solution.

pH is the negative logarithm of the activity of hydrogen ions in gram equivalents per litre. The pH value expresses the degree or intensity of both acidic and alkaline reactions on a scale from 0 to 14, with 7 representing neutrality, numbers less than 7 signifying increasingly greater acidic reactions, and numbers greater than 7 indicating increasingly basic or alkaline reactions.

Photoperiod is the duration of illumination and darkness within a 24-h day.

Precipitation is the formation of a solid (i.e., precipitate) from a solution.

Pre-treatment is, in this report, treatment of a sample or dilution thereof, prior to exposure of fish.

Salinity is the total amount of solid material, in grams, dissolved in 1 kg of seawater. It is determined after all carbonates have been converted to oxides, all bromide and iodide have been replaced by chloride, and all organic matter has been oxidized. Salinity can also be measured directly using a salinity/conductivity meter or other means (see APHA *et al.*, 1989). It is usually reported in grams per kilogram (g/kg) or parts per thousand (‰).

Turbidity is the extent to which the clarity of water has been reduced by the presence of suspended or other matter that causes light to be scattered and absorbed rather than transmitted in straight lines through the sample. It is generally expressed in terms of Nephelometric Turbidity Units.

Terms for Test Materials

Chemical is, in this report, any element, compound, formulation or mixture of a chemical substance that might enter the aquatic environment through spillage, application, or discharge. Examples of chemicals that are applied to the environment are insecticides, herbicides, fungicides, sea lamprey larvicides, and agents for treating oil spills.

Control is a treatment in an investigation or study that duplicates all the conditions and factors that might affect the results of the investigation, except the specific condition that is being studied. In an aquatic toxicity test, the control must duplicate all the conditions of the exposure treatment(s), but must contain no test material. The control is used to determine the absence of measurable toxicity due to basic test conditions (e.g., quality of the dilution water, health or handling of test organisms).

Control/dilution water means the water used for diluting the test material, or for the control test, or both.

Dechlorinated water means a chlorinated water (usually municipal drinking water) that has been treated to remove chlorine and chlorinated compounds from solution.

Deionized water is water that has been purified to remove ions from solutions by passing it through resin columns or a reverse osmosis system.

Dilution water is the water used to dilute a test material in order to prepare different concentrations for the various toxicity test treatments.

Distilled water is water that has been passed through a distillation apparatus of borosilicate glass or other material, to remove impurities.

Effluent is any liquid waste (e.g., industrial, municipal) discharged to the aquatic environment.

Elutriate is an aqueous solution obtained after adding water to a solid material (e.g., sediment, tailings, drilling mud, dredge spoil), shaking the mixture, then centrifuging or filtering it or decanting the supernatant.

Leachate is water or wastewater that has percolated through a column of soil or solid waste within the environment.

Receiving water is surface water (e.g., in a stream, river, or lake) that has received a discharged waste, or else is about to receive such a waste (e.g., it is just upstream from the discharge point). Further descriptive information must be provided to indicate which meaning is intended.

Reconstituted water is deionized or glass-distilled water to which reagent-grade chemicals have been added. The resultant synthetic fresh water is free from contaminants and has the desired pH and hardness characteristics.

Reference toxicant is a standard chemical used to measure the sensitivity of the test fish in order to establish confidence in the toxicity data obtained for a test material. In most instances, a toxicity test with a reference toxicant is performed to assess the sensitivity of the organisms at the time the test material is evaluated, and the precision of results obtained by the laboratory for that chemical.

Stock solution is a concentrated aqueous solution of the material to be tested. Measured volumes of a stock solution are added to dilution water in order to prepare the required strengths of test solutions.

Upstream water is surface water (e.g., in a stream, river, or lake) that is not influenced by the effluent (or other test material), by virtue of being removed from it in a direction against the current or sufficiently far across the current.

Wastewater is a general term that includes effluents, leachates, and elutriates.

Toxicity Terms

Acute means within a short period in relation to the life span of the organism, usually ≤4 days for fish. An acute toxic effect would be induced and observable within the short period.

Chronic means occurring during a relatively long period of exposure, usually a significant portion of the life span of the organism such as 10% or more.

Chronic toxicity implies long-term effects that are related to changes in such things as: metabolism, growth, reproduction, or ability to survive.

Chronic value is the geometric mean of the NOEC and LOEC in tests which have a chronic exposure. See also *TEC* as a recommended term.

Endpoint means the variables (i.e., time, reaction of the organisms, etc.) that indicate the termination of a test, and also means the measurement(s) or value(s) derived, that characterize the results of the test (NOEC, ICp, LC_{50}, etc.).

Flow-through describes tests in which solutions in test vessels are renewed continuously by the constant inflow of a fresh solution, or by a frequent intermittent inflow.

ICp is the inhibiting concentration for a (specified) percentage effect. It represents a point estimate of the concentration of test material that causes a designated percentage impairment in a quantitative biological function such as growth of fish. For example, an IC25 could be the concentration estimated to cause a 25% reduction in growth of larval fish, relative to the control. This term should be used for any toxicological test which measures a change in rate, such as reproduction, growth, or respiration. (The term EC_{50} or median effective concentration is limited to quantal measurements, i.e., number of individuals which show a particular effect.)

LC_{50} is the median lethal concentration, i.e., the concentration of material in water that is estimated to be lethal to 50% of the test organisms. The LC_{50} and its 95% confidence limits are usually derived by statistical analysis of mortalities in several test concentrations, after a fixed period of exposure. The duration of exposure must be specified (e.g., 7-d LC_{50}).

Lethal means causing death by direct action. Death of fish is defined as the cessation of all visible signs of movement or other activity.

LOEC is the lowest-observed-effect concentration. This is the lowest concentration of a test material to which organisms are exposed, that causes adverse effects on the organism, effects which are detected by the observer and are statistically significant. For example, the LOEC might be the lowest concentration at which growth of fish differed significantly from that in the control. LOEC is generally reserved for sublethal effects but can also be

used for mortality, which might sometimes be the most sensitive effect observed.

LT_{50} is the time (period of exposure) estimated to cause 50% mortality in a group of fish held in a particular test solution. The value is estimated graphically since there is no standard mathematical or computer technique in common use (see Appendix E).

NOEC is the no-observed-effect concentration. This is the highest concentration of a test material to which organisms are exposed, that does not cause any observed and statistically significant adverse effects on the organism. For example, the NOEC might be the highest test concentration at which an observed variable such as growth did not differ significantly from growth in the control. NOEC customarily refers to sublethal effects, and to the most sensitive effect unless otherwise specified.

Static describes toxicity tests in which test solutions are not renewed during the test.

Static renewal describes a toxicity test in which test solutions are renewed (replaced) periodically during the test, usually at the beginning of each 24-h period of testing. Synonymous terms are "batch replacement", "renewed static", "renewal", "static replacement" and "semi-static".

Sublethal means detrimental to the fish, but below the level that directly causes death within the test period.

TEC is the threshold-effect concentration. It is calculated as the geometric mean of NOEC and LOEC. *Chronic value* or *subchronic value* are alternative terms that might be appropriate depending on the duration of exposure in the test.

Toxicity is the inherent potential or capacity of a material to cause adverse effects on fish or other organisms. The effect could be lethal or sublethal.

Toxicity Identification Evaluation describes a systematic sample pre-treatment (e.g., pH adjustment, filtration, aeration) followed by tests for toxicity. This evaluation is used to identify the agent(s) which are primarily responsible for toxicity in a complex mixture. The toxicity test can be lethal or sublethal.

Toxicity test is a determination of the effect of a material on a group of selected organisms, under defined conditions. An aquatic toxicity test usually measures either (a) the proportions of organisms affected *(quantal)*, or (b) the degree of effect shown *(graded or quantitative)*, after exposure to specific concentrations of chemical, effluent, elutriate, leachate, or receiving water.

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Section 1

Introduction

1.1 Background

Aquatic toxicity tests are used within Canada and elsewhere to measure, predict, and control the discharge of materials that might be harmful to aquatic life. Recognizing that no single test method or test organism can be expected to satisfy a comprehensive approach to environmental conservation and protection, the Inter-Governmental Aquatic Toxicity Group (see Appendix A) proposed a set of aquatic toxicity tests which would be broadly acceptable, and would measure different toxic effects using organisms representing different trophic levels and taxonomic groups (Sergy, 1987). The test based on growth and mortality of larval fathead minnows was one of several "core" aquatic toxicity tests which was selected to be standardized sufficiently to help meet Environment Canada's testing requirements.

Universal procedures for a test with larval fathead minnows in the laboratory are described in this report. Also presented are specific sets of test conditions and procedures, required or recommended when using the test for evaluating different types of materials (namely samples of chemicals, effluents, elutriates, leachates, or receiving water) (Figure 1). Those procedures and conditions relevant to the conduct of the test are delineated and, as appropriate, discussed in explanatory footnotes.

In formulating these procedures, an attempt was made to balance scientific, practical, and financial considerations, and to ensure that the results will be accurate and precise enough for the majority of situations in which they will be applied. The authors assume that the user has a certain degree of familiarity with aquatic toxicity tests. Explicit instructions that might be required in a regulatory protocol are not provided, although the report is intended to serve as a guidance document useful for that and other applications.

1.2 Species Distribution and Historical Use in Tests

Fathead minnows belong to the family Cyprinidae, the carps and minnows, the dominant freshwater family in terms of number of species. Forty-four species of the family are found in Canada, most of them small minnows similar in appearance to the fathead minnow. Maximum lengths of fathead minnows in Canada are 8.3 to 9.4 cm, with mature females being smaller, normally 4 to 7 cm (Andrews and Flickinger, 1973; Scott and Crossman, 1973). A male fish of 7 cm would weigh about 3.5 to 5 g depending on nutritional status, and a female fish of 5 to 6 cm would weigh about 1.5 to 2 g (Benoit and Carlson, 1977; Korver and Sprague, 1989).

Fathead minnows *(Pimephales promelas)* are native to much of Canada. Their range touches the Northwest Territories (southern drainage of Great Slave Lake), covers most of Alberta, the southern two-thirds of Saskatchewan and Manitoba, most of Ontario (reaching Hudson Bay), southwestern Quebec, and the northwestern corner of New Brunswick. Moving southward through the United States, the range narrows to the central part of that country, and touches northern Mexico (Scott

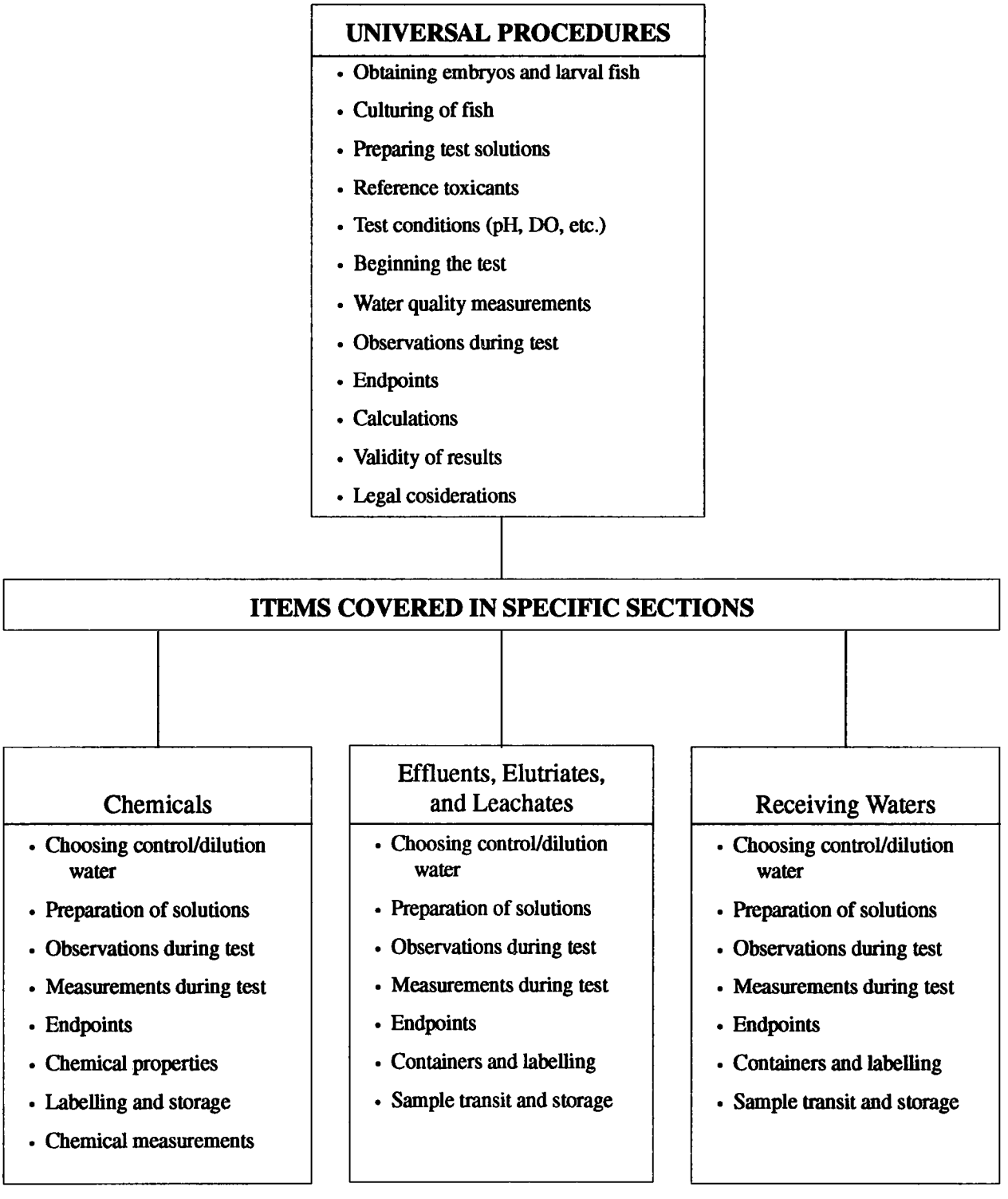

Figure 1 **Diagram of Approach Taken in Delineating Test Conditions and Procedures Appropriate for Various Types of Materials**

and Crossman, 1973). This fish is not native west of the Rocky Mountains, nor to the three most easterly Atlantic provinces, and a permit should be obtained before bringing the species to a laboratory in those places (see Section 2.2). The fathead minnow thrives in ponds, lakes, ditches, and slow muddy streams, and in alkaline or saline lakes such as those in Saskatchewan. It is an omnivore, feeding opportunistically on anything from living invertebrates to detritus, but is well-suited to a diet high in vegetable matter.

Fathead minnows will commence spawning in May or June in the northern part of their range when average water temperatures are as low as 13 to 17°C. Both temperature and photoperiod appear to play a role in initiating spawning, which could continue through the summer, ending by August or September (Andrews and Flickinger, 1973).

During the spawning season, the male fathead minnow selects an overhanging object (log or rock), cleans it, and defends it and the territory around it. Females are allowed to enter for spawning on the underside of the object, then the male continues to guard and clean the eggs. Cleaning is important in preventing fungus on the eggs. A female can produce 1000 to 10 000 eggs in a season depending on conditions, and could deposit 300 to 500 at one time. These characteristics make the species ideal for laboratory studies, because the male will adopt an inverted section of semicircular tile as a spawning territory, and the investigator can conveniently find and recover the embryos for counting or obtaining young fish. Fathead minnows are, in fact, good laboratory or aquarium fish, taking readily to that life and adapting well to dry commercial fish food, brine shrimp, etc. The species has been commonly reared in ponds in the U.S.A. for use as a bait fish (Brauhn *et al.*, 1975).

Fathead minnows have been used for life-cycle toxicity tests in the U.S. since the 1960s (Mount and Stephan, 1967), and it is now a standard species for tests of both acute lethality and sublethal or chronic effect (U.S. EPA, 1989). A toxicological data bank of appreciable magnitude has been assembled for this species.

The seven-day test with larval fish is a sensitive sublethal test, but is not of long duration relative to the life span of the fish, and is therefore not a chronic test. It might not estimate exactly, the results of longer exposures (Suter, 1990; Norberg-King, 1990). The seven-day test is sensitive, however, because larval fish are usually among the most vulnerable stages of the entire life cycle (reviewed in Woltering, 1984; McKim, 1985; Norberg and Mount, 1985; Suter *et al.*, 1987; Norberg-King, 1989). In general (from NOECs reported by Norberg-King, 1989[a]), the seven-day test could be expected to estimate the toxicity in

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[a] Norberg-King (1989) carried out many five- to seven-day larval tests with fathead minnows and also some 30- to 32-day exposures of early life-stages (starting with eggs). Those results were compared with each other and with the findings of five tests taken from the literature, for life-cycle exposures of fathead minnows with some of the same chemicals. The results are also discussed in Suter (1990) and Norberg-King (1990). Comparing the five- to seven-day larval exposures (24 tests using four chemicals) with ~30-day exposures, the latter tests showed somewhat greater toxicity. Average ratios of five- to seven-day NOEC to 30-day NOEC were: Carbaryl, 0.88; zinc, 1.3; cadmium, 2.3; and Diazinon, 8.6. For Dursban, a seven-day NOEC was compared to three, 30-day values from the literature, and the longer tests were more toxic by an average ratio of 1.8.

Comparing five- to seven-day larval NOECs with life-cycle NOECs, the latter showed appreciably greater toxicity. The ratios (seven-day NOEC divided by life-cycle NOEC) averaged 2.2 for zinc, 3.0 for chromium and Carbaryl, >25 for Dursban, and >45 for Diazinon. Suter *et al.* (1987) point out that fecundity of adults is usually the most sensitive effect in a life-cycle test, with larval growth and survival less sensitive and about equal in sensitivity to mortality of adults.

a 30-day exposure of early life-stages of fathead minnows closely in some cases, and within a factor of 2 in other cases, but it might sometimes under-predict by an order of magnitude. The seven-day test could underestimate the sublethal toxicity in a life-cycle exposure of fathead minnows by factors of 2 to 3 in many cases, but sometimes by factors of 25 or more. The larval test described in this report does not necessarily replace chronic toxicity tests, but comes much closer to results of such chronic tests than would a conventional lethality test with juvenile fish (e.g., Environment Canada, 1990a).

The seven-day larval test has shown excellent correlation with ecological evaluations of polluted waters. In a Kentucky river, the degree of mortality of larval fathead minnows had correlation coefficients of 0.92 to 0.96 with the number of fish species resident in sections of the river, and with the number of invertebrate species and their diversity (Birge *et al.*, 1989). McKim (1985) presents a rationale for use of tests with early life stages, and descriptions of these stages.

Precision of the seven-day test with larval fathead minnows has been satisfactory in the existing comparisons, for example there was good agreement in an intensive comparison among ten U.S. laboratories (API, 1988), with inter-laboratory coefficients of variation of 13% for survival of larvae and 52% for final weight. A coefficient of variation of 31% was shown for results from nine laboratories in the San Francisco area (Anderson and Norberg-King, 1991). That precision is somewhat better than in chemical analyses of priority pollutants, for which a comparable average inter-laboratory coefficient of variation was ≥60% (Rue *et al.*, 1988).

Fathead minnows are used in several Canadian aquatic toxicity laboratories, both governmental and industrial, for lethal and sublethal testing. A standard test method has been described in Ontario (Neville, 1989), but no standard method for the species has previously been published by a Canadian federal government agency.

In the United States, written descriptions of standard methods for sublethal tests have been provided by several groups. The most authoritative is from the Environmental Protection Agency (U.S. EPA, 1989), while other descriptions are essentially adaptations or abbreviated versions of the basic U.S. EPA procedure (e.g., Battelle, 1987; NJ, 1989).

The purpose of this document is to provide a "standardized" Canadian methodology for undertaking tests of sublethal toxicity of various materials using larval fathead minnows. The test procedures detailed in the U.S. documents vary in their coverage of endpoints, issues such as pH adjustment, differing methodology for various objectives, criteria for control/dilution water, and how to deal with samples that contain appreciable solids or floating material. This report is intended for evaluating sublethal toxicity of chemicals, effluents, leachates, elutriates, or receiving waters, and the rationale for selecting certain approaches is given.

The method is meant for use with freshwater-acclimated fish, with fresh water as the dilution and control water, and with effluents, leachates, or elutriates that are essentially fresh water (i.e., salinity ≤10 g/kg) or saline but destined for discharge to fresh water. Its application may be varied but includes instances where the impact or potential impact of materials on the freshwater environment is under

investigation. Other tests, using other species acclimated to seawater, may be used to assess the impact or potential impact of materials in estuarine or marine environments, or to evaluate wastewaters having a salinity >10 g/kg which are destined for estuarine/marine discharge.

Section 2

Test Organisms

2.1 Species and Life Stage

The test species is the fathead minnow (*Pimephales promelas*). Larvae that have been hatched for 24 hours or less must be used in tests[b].

2.2 Source

Breeding stock are best acquired from another laboratory that is known to have disease-free fish (Subsection 2.3.11). The least risk of carrying disease is by transfer of embryos, a procedure that also provides the greatest ease of shipment. Less desirably, fish may be acquired by collection in the field, but careful identification is required to separate this species from similar ones (Scott and Crossman, 1973). Parasites and disease are likely in wild fish, which should be carefully examined, reared in small isolated groups, and bred through a full generation before obtaining the next generation of progeny for use in tests (Brauhn *et al.*, 1975; Denny, 1987).

Procurement, shipment, and transfer of fish should be approved, if required, by provincial or regional authorities. Provincial governments may require a permit to import fish or their eggs whether or not the species is native to the area, or movements of fish stocks may be controlled by a Federal-Provincial Introductions and Transplant Committee. Advice on contacting the committee or provincial authorities and on sources of fish, can be obtained from the regional Environmental Protection office

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b Larvae that have been hatched for 24 h or less are used in tests because the very young fish are considered to be particularly sensitive, although there does not seem to be published evidence on this topic. Unpublished trials by the Duluth laboratory of U.S. EPA have apparently indicated that there can be decreased sensitivity among older larvae. Although fish of 1, 4, and 7 days age showed similar sensitivity to metals, the one-day-old fish were more sensitive to carbamate insecticides and other organic toxicants (personal communication, T.J. Norberg-King, U.S. EPA).

There would be one advantage in using older larvae. Some of them do not start feeding until 24 h after hatching, and perhaps about 6% of the larvae are in that category (API, 1988). Some of those larvae might never start to feed, in which case they would die within seven days and influence the results of the test or at least increase the variation in data obtained. By 48 h, larvae that are feeding can be distinguished by an orange colour of brine shrimp in the gut, and non-feeding larvae can be rejected, increasing the precision of the test. At present there does not appear to be quantitative information available, to permit an objective comparison of the relative importance of eliminating non-feeders, and the greater sensitivity of younger larvae.

The present method requires larvae of age ≤24 h in order to use more sensitive animals, and in order to increase the relevance and usefulness of data from tests conducted elsewhere, which will apparently be standardized on the 24-h age limit. The U.S. EPA method will continue to use fish of ≤1 day post-hatching (personal communication, T.J. Norberg-King), and in practice that means that all agencies and organizations in the U.S.A. will follow the procedure. The only exception in the U.S. EPA procedure is that larvae as old as 48 h may be used if they must be shipped to a remote site for the test (U.S. EPA, 1989), but that seems an unlikely prospect. It is clear that large amounts of test-data will be generated in the U.S. using larvae of age ≤24 h, and that data will be immediately useful for predictive purposes in Canada if the Canadian method is comparable. The method used by the Ontario Ministry of Environment uses larvae ≤24 h post-hatching (Neville, 1989).

(Appendix B). In areas where fathead minnows are not native (B.C., P.E.I., N.S., Newfoundland, and parts of other provinces and territories (see Section 1.2), application for a permit must be made to the above-mentioned committee, to the appropriate provincial agency, or to the Regional Director-General of the Department of Fisheries and Oceans (DFO), depending on procedures in place locally.

2.3 *Culturing*

2.3.1 General

The recommended conditions for holding and culturing fathead minnows, summarized in Table 1, are intended to allow some degree of flexibility within a laboratory, while at the same time standardizing those elements which, if uncontrolled, might affect the health of fish or viability of their offspring. Much of Section 2.3 that specifically concerns fathead minnows is derived from Denny (1987) and Norberg-King and Denny (1989), reports that should be consulted if further details are required.

A training video prepared by the U.S. Environmental Protection Agency (1988), shows procedures used by the Environmental Research Laboratory at Duluth, Minnesota for culturing fathead minnows. This video is available within Canada, courtesy of T. Norberg-King, U.S. EPA, Duluth), and can be obtained for viewing by contacting a regional office of Environment Canada (see Appendix B).

Small groups of male and female fathead minnows are held in aquaria provided with spawning substrates. The substrates are inspected daily, those with embryos are moved to hatching tanks, and new substrates are provided. Collections from the hatching tanks yield larvae, hatched for 24 hours or less, for use in tests. Some fish are reared as a source of new generations of adults. Two dozen pairs of spawning adults should provide at least 200 embryos per day on average, on a continuing basis if non-performing fish are periodically replaced with maturing fish, and 500 or more embryos per day under good conditions.

All larval fish used in a test must be from the same culture, and must be of known age. The larval fish should represent at least three spawnings (i.e., different parentage), although that is not an absolute requirement. It is strongly recommended that the culture of fathead minnows be maintained in the laboratory that carries out the toxicity tests. If necessary, however, embryos or larvae can be transported from another place, provided that all required characteristics of the water are maintained during that transfer.

2.3.2 Facilities

Embryos and larvae may be hatched and reared in containers made of nontoxic materials such as glass, stainless steel, porcelain, fibreglass-reinforced polyester, perfluorocarbon plastics (Teflon™), acrylic, polyethylene, or polypropylene.

Juvenile and adult fathead minnows may be reared in aquaria, troughs, or tanks that receive flowing water. These must also be made of nontoxic materials such as listed previously. Aquaria containing about 40 L of water, and provided with a standpipe drain, are most commonly used. The fish culture operation should be located away from any physical disturbances and preferably in a location separate from the test containers. Aquaria for rearing are usually indoors but may be outdoors; aquaria for obtaining embryos and young should be in the laboratory, exposed to the standard lighting, temperature, water, and other test conditions.

Table 1 Checklist of Recommended Conditions and Procedures for Culturing Fathead Minnows

Source of fish	- disease-free stock from another laboratory; captured in the wild if special care taken in identifying species and eliminating disease
Water	- uncontaminated ground, surface, or, if necessary, dechlorinated municipal water; flow to culture aquaria 1.4 L/g fish per day
Temperature	- holding temperature within the range 4 to 26°C; culturing at 25°C (range 23 to 26°) achieved at rate ≤3°C/d and held at 23 to 26°C for ≥2 weeks
Oxygen/aeration	- dissolved oxygen 80 to 100% saturation; maintained by aeration with filtered, oil-free air if necessary
pH	- within the range 6.8 to 8.5, preferably 7.0 to 8.5
Water quality	- temperature, dissolved oxygen, pH, and flow to each holding or culturing aquarium to be monitored, preferably daily
Lighting	- at water surface, ≤500 lux, with 16 ± 1 h light:8 ± 1 h dark, preferably with gradual transition, and preferably from full-spectrum fluorescent lights
Feeding	- at least once a day with frozen brine shrimp supplemented by commercial pelleted or flaked food; feeding rate judged by amount consumed in 10 minutes; food stored as recommended by manufacturer
Cleaning	- siphoning of debris, daily or as required
Disease	- mortalities monitored daily and moribund fish removed; mortality ≤5% during seven days preceding collection of embryos; special measures if ≥10%/week among adult/juvenile stock; groups of diseased fish should be discarded; if they must be kept and chemically treated for disease, allow at least four weeks before collecting eggs for use in toxicity tests

Breeding aquaria are divided or partially divided for spawning purposes with stainless steel screens or rigid plastic sheets, opaque or transparent. A spawning substrate, intended as territory for one male minnow, is placed in each of the areas created. There are variations in the exact arrangements which are not crucial. A 40-L aquarium might have two areas, with a spawning substrate in each, and a partial divider with an open "doorway" in it for fish (essentially the females) to move back and forth. Alternatively, the aquarium might be divided into four areas, intended for four substrates with a male and female in each area (Denny, 1987).

The spawning substrate is one half of a cylinder of tile or pipe. The material is not critical and could be PVC plastic, or a porous material such as clay or concrete (Benoit and Carlson, 1977). Tile of about 10 cm diameter is cut into lengths of 7 to 10 cm, then cut in half lengthwise. One half is inverted in the area intended for each male.

White plastic dishpans are convenient "hatchery trays" if the embryos are hatched on the tiles. Up to six such trays can stand partly immersed in a large water bath. If that bath is under the breeding aquaria, wastewater from those aquaria can flow to the bath, providing the basic heating of the water bath. Alternatively, the eggs may be removed from the tiles and hatched in a separatory funnel (Subsection 2.3.8).

2.3.3 Lighting

Lighting during rearing and breeding may be natural or provided by overhead fixtures, preferably supplying full-spectrum fluorescent lights[c]. Photoperiod should normally be a constant sequence of 16 hours of light and 8 hours of darkness. A light intensity of 500 lux, as measured at the water surface, will provide adequate illumination for rearing fish. A 15- to 30-minute transition period between light and dark is recommended if artificial lighting is provided[d].

2.3.4 Water

Sources of water for holding and culturing fish can be "uncontaminated" groundwater, surface water, or if necessary, dechlorinated municipal drinking water (see the following paragraph). The water supply should previously have been demonstrated to consistently and reliably support good survival, health, and growth of fathead minnows. Monitoring and assessment of variables such as residual chlorine (if municipal water is used), pH, hardness, alkalinity, total organic carbon, conductivity, suspended solids, dissolved oxygen, total dissolved gases, temperature, ammonia nitrogen, nitrite, metals, and pesticides, should be performed as frequently as necessary to document water quality.

Dechlorinated water is not recommended for culturing of fish and, in particular, not for hatching of embryos or rearing of larvae. It is difficult to remove the last traces of residual chlorine and chlorinated organic substances, and they could be toxic to the larval fathead minnows. If municipal drinking water is to be used for culturing fish and as control/dilution water, effective dechlorination must rid the water of any harmful concentration of chlorine. Vigorous aeration of the water can be applied to strip out part of the volatile chlorine gas. That could be followed by use of activated carbon (bone charcoal) filters and subsequent ultraviolet radiation (Armstrong and Scott, 1974) for removing most of the residual chloramine and other chlorinated organic

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[c] It is recommended but not required, that any artificial lighting should be supplied by fluorescent lamps with a full-spectrum wavelength (e.g., Vitalite™, Benelux 55™), to simulate natural lighting characteristics (Denny, 1987). Various laboratories report success in rearing fathead minnows with the less expensive cool white or warm white fluorescent lighting.

[d] A "dawn/dusk" transition period is recommended because abrupt changes in intensity startle and stress fish. Automated control systems are available for dimming and brightening the intensity of fluorescent lights, although they are costly. Alternatively, a secondary incandescent light source, regulated by time clock and automated rheostat, may be used to provide the transition period.

compounds. Aging the water in aerated holding tanks might be of further benefit. A target value for total residual chlorine, recommended for the protection of freshwater aquatic life, is ≤0.002 mg/L (CCREM, 1987). Anything greater than 0.002 mg/L might risk interaction of chlorine toxicity with whatever was being tested[e]. In addition to measurements of chlorine, monitoring of egg production and fish survival can provide evidence of satisfactory water.

If surface water is used for culturing fish, it should be filtered to remove potential predators and competitors of fathead minnows. A conventional sand filter or commercial in-line filter would be suitable. Small quantities might be filtered through a fine-mesh net (≤60 µm). Ultraviolet sterilization is recommended to reduce the possiblity of introducing pathogens into the colony of fish.

If reconstituted water is to be used as dilution and control water (see Section 5.3), adult fish must be acclimated to that reconstituted water or to a similar water, for at least the five days immediately before embryos are obtained for the test[f]. The similar water could be: (a) a natural water with hardness within 20% of the reconstituted water; (b) a harder natural water adjusted downwards to the desired hardness with deionized water; or (c) a softer natural water adjusted upwards with the appropriate quantities and ratio of reagent-grade salts (e.g., ASTM, 1980; Environment Canada, 1990b, Table 2).

The water in aquaria containing adult fish should be renewed to prevent a buildup of metabolic wastes. At least 1 mL/min of fresh (new) water should flow into the tank for every gram of fish being held (equals 1.4 L/g fish•d or 0.69 g fish•d/L)[g]. For an aquarium with 50 g of fish, that would be an inflow of 70 L/d or 50 mL/min. Unusual circumstances such as acclimation of fish to reconstituted water might require the filtration and recirculation of water, or its periodic renewal in static systems. A recirculating culture system is described by Rottmann and Campton (1989). In such cases of water reuse, ammonia and nitrite should be measured frequently to check that they do not reach harmful levels. Target values, recommended for the protection of freshwater aquatic life, are ≤0.02 mg/L of

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e Chlorine is quite toxic to aquatic organisms, especially to crustaceans and the early life-stages of fish. For example, 0.011 mg/L of total residual chlorine causes chronic sublethal effects in fathead minnows, and somewhat higher concentrations of 0.040 to 0.045 mg/L might cause acute lethality of rainbow trout and a species of minnow (Arthur and Eaton, 1971; Wolf *et al.*, 1975; Ward and DeGraeve, 1978; CCREM, 1987). An exposure to 0.08 mg/L of total residual chlorine for only 2 h/d might prove lethal to juvenile fathead minnows (Wilde *et al.*, 1983), suggesting that sublethal effects could be expected at concentrations at least an order of magnitude lower. The recommended limit of 0.002 mg/L taken from Canadian guidelines (CCREM, 1987) is in line with recommendations in the United States. A maximum of 0.002 mg/L was recommended for protection of most aquatic life in receiving waters by Brungs (1973), and a maximum of 0.003 mg/L at any time or place was recommended for freshwater life by NAS/NAE (1974).

f Without such acclimation, the benefit of a standardized dilution water might be lost. For example, it takes several days for fish to readjust their tolerance to heavy metals when moved to a water of different mineral content (Lloyd, 1965).

g If necessary (e.g., if fish are being acclimated to reconstituted water, receiving water, or some other water source that is restricted in amount), water-volume requirements for fish acclimation may be decreased substantially by recirculating the flow to the fish tank through a filter suitable for removing metabolic wastes (e.g., Rottmann and Campton, 1989). If a recirculation system is used, ammonia and nitrite concentrations in the water should be monitored and kept below levels harmful to fish health.

un-ionized ammonia (OME, 1984), and ≤0.06 mg/L of nitrite (CCREM, 1987).

Water entering the aquaria must not be supersaturated with gases. In situations where gas supersaturation within the water supply is a valid concern, total gas pressure within water supplies should be frequently checked (Bouck, 1982). Remedial measures must be taken (e.g., use of aeration columns or vigorous aeration in an open reservoir) if dissolved gases exceed 100% saturation. It is not a simple matter to completely remove supersaturation, and frequent checking should be done if the problem is known or suspected to exist.

Water temperature, dissolved oxygen, pH, and flow should be monitored for each aquarium or tank, preferably daily. Weekly or more frequent monitoring of levels of ammonia, nitrite, and total residual chlorine (if municipal water source) is recommended.

2.3.5 Temperature

Groups of fish may be held for later use at temperatures as low as 4°C. High temperatures should be avoided, the optimum for the species being 23.5°C, and 32°C marking a limit for failure of reproduction and the onset of effect on growth (Brungs, 1971b). When preparing a group of fish for breeding, water temperature may be changed at a rate not exceeding 3°C per day, until a value near 25°C is achieved. Fish should be maintained within the range 22 to 26°C for a minimum of two weeks and preferably ≥3 weeks, before using their embryos to obtain larvae for toxicity tests. Temperatures outside the 22 to 26°C range are known to decrease egg production (Brungs, 1971b).

2.3.6 Dissolved Oxygen

The dissolved oxygen (DO) content of the water within holding and culture aquaria should be 80 to 100% air saturation. Mild aeration of the tanks should be carried out using filtered, oil-free compressed air. Such aeration through a commercial aquarium airstone is customary and assists in mixing the water and maintaining uniform physicochemical conditions. Avoid vigorous aeration, especially if larval or young fish are present.

2.3.7 pH

The pH of water used for holding and culturing fish should be within the range 6.8 to 8.5, and preferably[h] 7.0 to 8.5.

2.3.8 Growing and Breeding the Fish

Post-larval fish, juveniles, and maturing fish are normally reared in aquaria. The number per aquarium should be gradually reduced as they grow, by moving fish to other aquaria. As the fish approach adult size, males and females can be obtained for stocking the breeding aquarium. The sexes can be distinguished only as the fish approach breeding condition, usually at about 16 to 24 weeks of age (Figure 2). Females retain the appearance of a silvery minnow but develop an ovipositor ahead of the anal fin. Males are bigger, become blackened on the sides with two light-coloured vertical bars near the front of the body, and develop a pad with tubercles on the back part of the head and tubercles on the "forehead" or snout. Two or three spawning substrates should be placed in the culturing aquaria as maturation becomes evident, and some males will claim them. They stay under the shelter (i.e., the tile or other spawning substrate) most of the time and keep other fish away, except for the purpose of spawning.

Upon signs of maturation, individual males and females may be selected from the culture tank and used to stock the breeding aquaria. Other males will eventually take over the substrates and other females will develop, and more breeding aquaria can be stocked. If a breeding aquarium is divided into two areas, it might be stocked with two males and four to six females. Alternatively, one male and one female might be placed in each of four screened-off areas in an aquarium; Denny (1987) indicates that the paired method increases total egg production and points out that it allows good records of egg production, so that non-productive fish can be replaced. Fish should be replaced with others if there is a three-week period without egg production. Automatic replacement of fish could be practised, after a fixed period of three or six months.

Eggs will be laid inside the tile substrate on the "ceiling". Daily inspection should be made at mid-morning because spawning often takes place in early morning. Inspection may be done by feeling inside the tile with a finger, or less desirably, removing and inspecting the tile. If there are embryos, the tile is removed and replaced with a clean one. The tile with embryos is moved to a hatching tray. Two such tiles might be placed on end in a circle, with an airstone inside to keep the water moving. To help prevent the spread of fungal infections it is desirable to aerate tiles individually in beakers immersed in a hatching tray (Subsection 2.3.2).

Incubating embryos must be inspected daily. Dead embryos or those with fungal growth must be removed and discarded, the dead

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h The objective in holding and culturing is to provide conditions that are quite favourable for the fish. Fathead minnows are not well adapted to acidic pH values. There is evidence of marginal effects of acidic conditions, up to pH 6.6, hence the recommended minimum of pH 6.8 for culture of fish. Maintaining pH 7.0 or higher is considered a more realistic margin of safety to avoid any sublethal effects during culture. That pH is therefore preferred, although a scarcity of data in the region between pH 6 and 7 do not provide much direct support for the limit.

A pH of 6.0 is clearly unsatisfactory for reproduction of fathead minnows and appears unsatisfactory for rearing the species. In soft water in the laboratory, McCormick *et al.* (1989) demonstrated an appreciable reduction in survival from spawned egg to 4 days' posthatch, at pH 6 compared to pH 7.5. At pH 7.5, 6, and 5.5, survivals were respectively 92%, 60%, and 0%, with a trace level of aluminum present that should not have affected results. In a long experiment in an outdoor artificial stream (one of the most meaningful types of toxicity test), Zischke *et al.* (1983) found that at pH 8 (control), pH 6, and pH 5, the estimated numbers of eggs produced by parallel populations of fathead minnows were respectively 41 000, 33 100, and 110, while the numbers of young surviving at the end of the experiment were respectively 2924, 14, and 1. Zischke and his co-authors conclude that "Continued acidification would likely have caused extinction of the fathead minnow populations at both pH 5 and pH 6. Our results ... give support to the proposed protection pH recommendations (6.5 or higher) set by the National Academy of Sciences (1973) and the U.S. Environmental Protection Agency (1976)" [NAS/NAE, 1974; and U.S. EPA, 1976].

One Canadian laboratory had severe difficulties in testing with larval fathead minnows at pH 6.5 and suffered control mortalities severe enough that some tests had to be repeated six or more times (pers. com., T. Kovacs, Pulp and Paper Research Institute of Canada, Pointe Claire, Que.). Mount (1973) did a life-cycle test with fathead minnows in hard water which would be favourable to the fish (hardness = 200 mg/L), with results that indicated some deleterious effects on reproduction at pH 6.6. For exposures at four values of pH, Mount (1973) recorded the following numbers of eggs produced per female in two replicates: pH 7.5 = 480 and 968; pH 6.6 = 210 and 394; pH 5.9 = 66 and 101; and pH 5.2 = 0 and 0. The number of spawnings per female in the two replicates were respectively, for the same four values of pH: 4.2 and 5.4; 3.0 and 4.0; 1.0 and 1.2; 0 and 0. Statistical analysis of Mount's results cannot be done without access to the raw data, but the reduction of average number of eggs at pH 6.6, to about 42% of the number at pH 7.5, has the appearance of a deleterious effect.

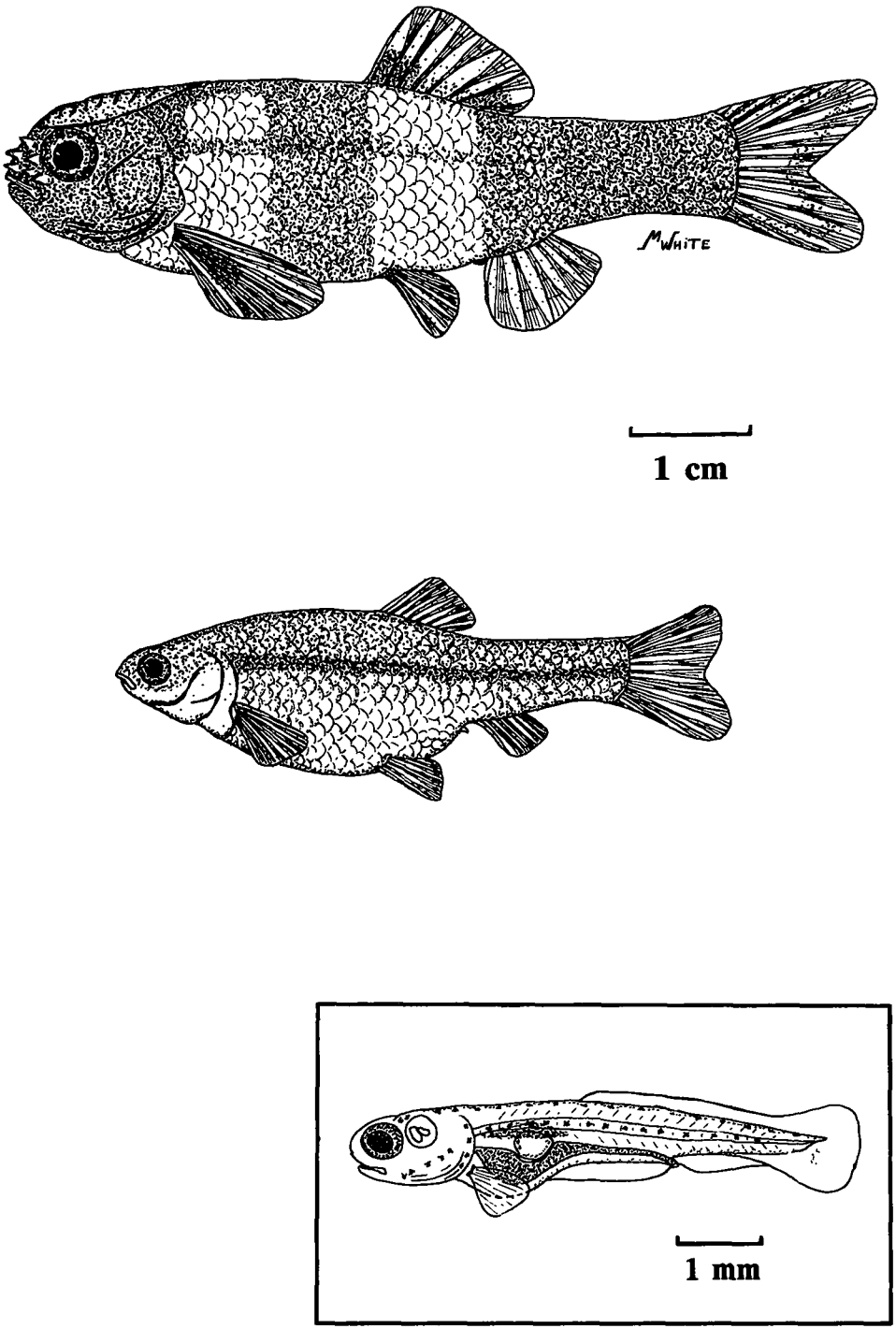

Figure 2 **General Appearance of Male and Female Fathead Minnows in Breeding Condition, and of a Larva About Four Days After Hatching**
(original drawings from specimens, by M.A. White)

Non-breeding males would not have the tubercules on the snout, the rugose pad on the top of the head, or the vertical bands of colour. Non-breeding females would not show the ovipositor ahead of the anal fin, nor the ventral distension from carrying eggs.

ones appearing opaque or having a white spot inside. Tiles with severe fungal growth should be removed. Disturbance should be minimal on days 3 to 5 because it might cause early hatching. Embryos will hatch in 4 to 5 days depending on temperature, which should be maintained in the 22 to 25°C range. Larvae are then removed with a large-bore pipette with rubber bulb, and used for tests. The used spawning tiles are disinfected (Subsection 2.3.10) and thoroughly soaked and rinsed in water before reuse. To culture fish towards adulthood for a new stock of breeders, groups of 200 to 300 larvae are placed in aquaria with a water depth of 20 cm. For these groups of fish being cultured, there should be an approximate estimate of hatching success, and mortality among the ensuing larvae during the first 30 days of life.

An alternative technique for culturing is to gently roll the eggs off a smooth spawning tile (PVC) using a wet finger. Eggs are placed in a separatory funnel with aeration to keep them suspended in the water. Dead eggs or those with fungal growth are removed and discarded at 24 and 48 hours. At 48 h, viable eggs are moved to small pans or aquaria and vigorously aerated until hatching.

If there is long-continued culture of minnows in a laboratory, steps should be taken to avoid selecting a homogenous strain. Larvae for future spawning stock should be selected from different parents at intervals, rather than keeping many larvae from a few spawnings or a single spawning. Every two years, the gene pool might be supplemented by exchanging with another laboratory; however, bringing in some wild fish would be preferred. The new fish should be carefully examined by a taxonomic expert. Any diseased fish should be rejected and the rest treated for disease (Subsections 2.3.10 and 2.3.11; Denny, 1987), kept segregated in small groups, and held through breeding so that it is actually their progeny that are added to the laboratory stock. It is encouraging that when ten laboratories engaged in round-robin studies, source of fish did not appear to affect results (API, 1988).

2.3.9 Feeding

It is recommended that juvenile and adult fathead minnows be fed with frozen brine shrimp, supplemented with other commercial fish food. Commercial "flake" food may be used for part of the diet but only as a supplement to the frozen brine shrimp. Commercial pelleted fish food of suitably small size may also be used, again as a supplement. Depending on water temperature and fish size, feeding should be one or more times daily, normally with a daily ration approximating 1 to 5% of wet body weight. In practice, the amount of food required is best judged by the amount the fish consume in about 10 minutes, the amount left over on the bottom of the tank, and by the appearance and condition of the fish. The method and maximum duration for storing fish food should be as recommended by the manufacturer.

Newly hatched fish should be fed with nauplii of brine shrimp *(Artemia salina)* that have been hatched from embryos within the previous 24 hours (Appendix C). Fathead minnow larvae begin feeding toward the end of their first day of life or shortly thereafter, so feeding could start when they are about 12 hours old. Brine shrimp should be supplied at least twice a day since they might live in fresh water only about 8 hours. The first daily feeding of the minnow larvae should be early in the day, so that live nauplii are always available during the daylight hours. Fish in the early larval stages can ingest only small, newly hatched

nauplii of brine shrimp (maximum size 0.24 to 0.28 mm). As the larvae grow they can be fed larger brine-shrimp nauplii, and, after about 30 days, can be gradually weaned from living to frozen brine shrimp and supplements of other food. The amounts to be fed (volume of fluid containing concentrations of nauplii) depend to some extent on the nature of the rearing system (Appendix C).

It is desirable to assess toxic contaminants in all fish food, but particularly dry flake food and brine-shrimp eggs (Appendix C). Toxicants of concern are bioaccumulative metals and pesticides. Guidance can often be obtained from the experience of other laboratories, and the measurements that they have done. It is desirable to report the origin of the brine shrimp eggs so that any association between the source and success in rearing and testing can be detected over time.

2.3.10 Cleaning of Tanks

Tanks used to hold and culture fish should be kept reasonably clean. Excess food and faeces should be siphoned out with minimum disturbance of fish, once a day or as frequently as necessary to eliminate a buildup. Excessive growths of fungi or blue-green algae should be scraped and removed, and an effort made to eliminate whatever conditions are favouring their growth. However, a light growth of other algae and invertebrates on the walls of the aquaria should be tolerated because that might provide supplementary food and activity for the fish.

To minimize the occurrence of disease, tanks should be disinfected before introducing a new batch of fish. Suitable disinfectants include those containing chlorinated or iodophore compounds or n-alkyldimethyl-benzylammonium chloride (e.g., Comet™, Ovidine™, Argentyne™, Roccal™). As disinfectants are toxic to fish, tanks should be rinsed thoroughly with the water used for culturing fish, following disinfection.

2.3.11 Fish Morbidity, Mortality, and Treatment

Adult and pre-adult fish being cultured should be inspected daily for signs of disease (Amlacher, 1970; Brown and Gratzek, 1980; Roberts and Shephard, 1986)[i]. Dead and moribund individuals should be removed immediately. During the seven-day period preceding the collection of eggs, mortalities should be less than 5% of the general population being reared, and less than 5% of the fish in individual tanks or aquaria, or limited to one fish in the case of breeding aquaria containing small numbers of fish. If mortality exceeds those limits, holding of fish should be extended for at least another seven days before collection of eggs, until no more than 5% mortality in seven days is realized. If mortalities of the adult breeding stock exceed 10% per week at any time[j], that stock of fish should not be used to produce test fish, an intensive search for the cause of mortality should be carried out, and cultures should be started anew from apparently healthy stock.

Treatment of diseased fish with chemicals should be avoided if possible; it is strongly

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i Symptoms of unhealthy fish include loss of appetite, abnormal distribution in the tank, lethargy, erratic or atypical swimming behaviour, darkened coloration, pale gills, eroded or frayed fins, and external lesions. Books by Amlacher (1970) and Brown and Gratzek (1980) are useful guides for preliminary identification and diagnosis of fish diseases. *The Handbook of Trout and Salmon Diseases, 2nd ed.* (Roberts and Shephard, 1986) is useful for the same purposes for a variety of fishes, as well as salmonids.

j Based upon mortality criteria specified by the Organization for Economic Cooperation and Development (OECD, 1984).

recommended that groups of fish showing signs of disease be discarded. That might be a feasible approach if groups are held separately in a number of aquaria or tanks. If fish are treated, a minimum four-week period should follow before their eggs are collected for tests.

Section 3

Test System

3.1 Facilities and Apparatus

The test is to be conducted in a facility isolated from general laboratory disturbances. If a separate room is unavailable, the test area should be surrounded with an opaque curtain (e.g., black plastic) to minimize stress to fish during testing. Dust and fumes should be minimized within the test and culturing facilities.

A test facility is required that will maintain the temperature of all test solutions at a mean temperature of 25 ± 1°C with extreme fluctuations within the range 23 to 27°C. This may be achieved using various types of equipment such as a thermostat-controlled air conditioning unit or a temperature-controlled water bath in which test vessels are immersed.

Construction materials and any equipment that may contact the test solutions or control/dilution water should not contain any substances that can be leached into the solutions or increase sorption of test material (see Subsection 2.3.2). The laboratory must have the instruments to measure the basic water quality variables (temperature, conductivity, dissolved oxygen, pH), and must be prepared to undertake prompt and accurate analysis of other variables such as hardness, alkalinity, ammonia, and residual chlorine.

3.2 Lighting

Lighting conditions should be the same as those defined in Subsection 2.3.3. The photoperiod is to be timed to coincide with that at which the parent fish were held.

3.3 Test Vessels

Vessels may be beakers or rectangular containers of borosilicate glass[k] (such as Pyrex™), perfluorocarbon plastics (Teflon™), or disposable polystyrene. Nontoxic containers of other plastic such as polypropylene or polyethylene may be used, but should not generally be reused in a second test, in case the plastic sorbs toxicants that could be released during a subsequent test.

The vessel must contain at least 250 mL of solution during the test, and 500 mL is recommended. Volumes of up to 1L are suitable and would give additional protection against depletion of toxicant or dissolved oxygen. The minimum water depth in any test vessel should be 3 cm. The vessel should not unduly restrict the surface area of the test solution, because diffusion of oxygen through the surface could be important when testing effluents or other materials with an oxygen demand. As a guideline, the diameter of the vessel should approximate the depth of the test solution. Using that guideline, 500 mL of liquid should fill a container of 8.6 cm diameter to

k Glass containers are inert and easily cleaned, and permit the unimpeded observation of test fish. Adsorption to non-glass containers (e.g., polyethylene, polypropylene, stainless steel, etc.) is markedly different for certain chemicals.

a depth of 8.6 cm, yielding a surface area of about 58 cm^2.

Considerable latitude is allowed in the design and shape of test vessels. They may be specially constructed for easy renewal of test solutions without damaging the fish. For example, a screened sump at one end of a glass container can be used to remove the old test solution by siphoning, with larvae held safely on the other side of the screen (Norberg and Mount, 1985). (That method still requires dislodging and siphoning debris directly from the area containing larvae.) Alternatively, plastic containers with a screened bottom or other types of mesh "cages" can be submerged in a larger vessel of test solution and lifted easily to another vessel with fresh solution. Nytex™ netting of 500 µm size has been found advantageous since dead brine shrimp can pass through it but the fish larvae will be retained. For a given test, water depth and container type, size and shape should be identical for each test solution. Vessels should be covered with glass during the test to avoid potential contamination from the air and loss of volatile components.

3.4 Control/Dilution Water

Depending on the test material and intent (Sections 5 to 7), the control/dilution water may be: "uncontaminated" groundwater or surface water from a stream, river, or lake; reconstituted water of a desired pH and hardness (e.g., simulating that of the receiving water); a sample of receiving water collected upstream of the source of contamination, or adjacent to the source but removed from it; or dechlorinated municipal water[1] (see Subsection 2.3.4). Conditions for the collection, transport, and storage of samples of receiving water should be as described in Subsection 6.1. If surface water is used, it should be filtered through a fine-mesh net (≤60 µm) to remove potential predators and competitors of fathead minnows.

The control/dilution water must be adjusted to 25 ± 1°C before use. This water must not be supersaturated with excess gases (see Subsection 2.3.4). Before it is used, control/dilution water should have a dissolved oxygen content that is 90 to 100% of the air-saturation value. If necessary, aerate it vigorously (oil-free compressed air passed through air stones) immediately before use, and confirm that dissolved oxygen levels representing 90 to 100% saturation have been achieved.

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1 The addition of thiosulphate or other chemicals to dilution water in order to remove residual chlorine is not recommended. Such chemical(s) could alter sample toxicity.

Section 4

Universal Test Procedures

Procedures described in this section apply to all the tests of particular chemicals and wastewaters described in Sections 5, 6, and 7. All aspects of the test system described in Section 3 must be incorporated into these universal test procedures. The summary checklist of recommended conditions and procedures in Table 2 includes not only universal procedures but also those for specific types of test materials.

4.1 Preparing Test Solutions

All test vessels, measurement devices, stirring equipment, and fish-transfer pails must be thoroughly cleaned and rinsed in accordance with standard operational procedures. Control/dilution water should be used as the final rinse water.

For tests that are intended to estimate the NOEC/LOEC and/or ICp, at least five concentrations plus a control solution (100% dilution water) are to be prepared[m]. An appropriate geometric dilution series may be used, in which each successive concentration is about 50% of the previous one (e.g., 100, 50, 25, 12.5, 6.3). Concentrations may be selected from other appropriate logarithmic series (see Appendix D). There is not usually a great improvement in precision of the test from the use of steps smaller than the 50% dilution factor (i.e., concentrations closer together). A dilution factor as low as 30% (e.g., concentrations 100, 30, 9, etc.) is not recommended for routine use because of poor precision of the estimate of toxicity; however, it might be used if there is considerable uncertainty about the range of concentrations likely to be toxic.

In cases of appreciable uncertainty about sample toxicity, it is beneficial to run a range-finding or screening test for the sole purpose of choosing concentrations for the definitive test. Conditions and procedures for running the test can be relaxed. A 24-h exposure to determine mortality of larvae at a wide range of concentrations (≥2 orders of magnitude) should be of assistance in selecting concentrations for the full test. The highest concentration for the definitive test should be one that did not cause more than 20 to 30% mortality in the range-finding test. If there are severe time-limitations on starting the definitive test, a range-finding test of shorter duration such as 8 h would still provide useful guidance.

Single-concentration tests could be used for regulatory purposes (e.g., pass/fail). They would normally use full-strength effluent, elutriate, leachate, or receiving water, or an arbitrary or prescribed concentration of chemical. Use of controls would follow the same rationale as multi-concentration tests. Single-concentration tests are not specifically described here, but procedures are evident, and all items apply except for testing a single concentration and a control.

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[m] A preliminary or range-finding test may be conducted before starting the definitive test. A range-finder normally covers a broader concentration range, and is frequently terminated in 24 h or less.

Table 2 Checklist of Recommended Test Conditions and Procedures

Universal	
Test type	–renewed static, 7-d duration*
Control/dilution water	–ground, surface, or if necessary, dechlorinated municipal water; "upstream" water to assess toxic impact at a specific location**; reconstituted water if requiring a high degree of standardization; dissolved oxygen (DO) 90 to 100% saturation at time of use
Fish	–larval fathead minnows hatched for ≤24 h; at least ten larvae in each of three replicate test vessels required at each concentration, and four replicates recommended
Vessel/solution	–depth ≥3 cm, and ≈ diameter; volume ≥250 mL, preferably 500 mL
Temperature	–daily mean 25 ± 1°C with extreme fluctuations within the range 23 to 27°C
Oxygen/aeration	–no pre-aeration unless a test solution has DO <40% or >100% saturation upon preparation, in which case aerate all test solutions for ≤20 minutes at minimal rate before starting test or renewing solution; DO 40 to 100% saturation throughout the test, with more frequent renewal if required to maintain DO; if necessary to meet objectives of test, gentle aeration of all vessels
pH	–no adjustment if pH of test solutions is in range 6.5 to 8.5***; a second (pH-adjusted) test might be required or appropriate, for pH beyond that range, or at any pH below 7.0
Lighting	–at water surface, ≤500 lux, with 16 ± 1 h light:8 ± 1 h dark, preferably with gradual transition and preferably supplied by full-spectrum fluorescent lights
Feeding	–two or three times/day with newly-hatched brine shrimp nauplii; feed at the start of the test but do not feed during the final 12 h
Observations	–mortality, swimming behaviour, every 24 h; mean dry weight at 7.0 d
Measurements	–temperature, pH, and DO at start and end of 24-h periods, representative concentrations; conductivity at least at start of 24-h periods; hardness of control/dilution water and highest concentration at start of test
Endpoints	–NOEC/LOEC and/or ICp for growth, mortality; if appropriate, LC_{50} at selected time(s).
Reference toxicant	–sodium chloride, phenol and/or zinc; test for NOEC/LOEC and/or ICp, monthly

Test validity	–invalid if >20% of control fish die or exhibit clearly atypical swimming behaviour, or if average weight of control fish is not ≥250 μg; validity and usefulness of test is questionable if the Minimum Significant Difference of weights is >20% of the mean control dry weight
Chemicals	
Solvents	–used only in special circumstances; maximum concentration, 0.1 mL/L
Concentration	–recommended measurements are at beginning and end of 24-h renewal periods, in high, medium, and low strengths and control(s); if concentrations decline ≥20%, re-test with more frequent renewal or flow-through methods
Control/dilution water	–as specified and/or depending on intent; reconstituted water if high degree of standardization required; receiving water if concerned with local toxic impact; otherwise, laboratory water
Effluents, Leachates, and Elutriates	
Sample requirement	–for off-site tests, at least three samples are collected or prepared (elutriates), and used as indicated in Section 6.1; for on-site tests, samples are collected daily, and used within 24 h; a 4-L volume is adequate
Transport and storage	–if warm (>7°C), cool to 1 to 7°C with ice or frozen gel packs; transport at 1 to 7°C (preferably 4 ± 2°C) using frozen gel packs as necessary; sample must not freeze during transit; store in the dark at 1 to 7°C (preferably 4 ± 2°C); use in testing should begin within 24 h and must start within 72 h of sampling/extraction
Control/dilution water	–as specified and/or depends on intent; laboratory water or "upstream" receiving water for monitoring and compliance
High solids	–second test with filtered sample is an option, to assess effects of solids
Receiving Water	
Sample requirement	–as for effluents, leachates, and elutriates
Transport, storage	–as for effluents, leachates, and elutriates
Control/dilution water	–as specified and/or depends on intent; if studying local impact use "upstream" receiving water as control/dilution water

* Special situations (e.g., volatile or unstable chemicals in solution) may require the use of flowthrough tests.

** For this option, there must be an additional control using the laboratory water in which fish were cultured.

*** If pH is outside this range or below pH 7.0, results may reflect toxicity due to biologically adverse pH.

There must be at least three replicates of each concentration including controls*. They are required for the statistical analysis of results, specifically they are required by Dunnett's test (Gulley *et al.*, 1989). It is recommended but not absolutely required that four replicates should be used, because that many would be required for non-parametric statistical analysis, if results of the test did not satisfy requirements for normality and homogeneity. The test must start with an equal number of replicates for each concentration including controls. If there is accidental loss of a replicate during the test, unbalanced sets of results can be analyzed with less power (Gulley *et al.*, 1989).

When receiving water is used as control/dilution water, a second control solution should be prepared using the laboratory water in which fish were kept for production of embryos, and in which the embryos hatched into larvae. Upstream water cannot be used if it is clearly toxic according to the criteria of the test for which it was intended[n]. In such cases, the laboratory water used for breeding should be used as the control/dilution water.

The same control/dilution water must be used for preparing the control and all test concentrations. Each test solution should be well mixed with a glass rod, Teflon™ stir bar or other nonreactive device. Temperatures should be adjusted as required to 25 ± 1°C. It might be necessary to adjust the pH of the sample of test material or the test solutions (see Subsection 4.3.2), or to provide preliminary aeration of the test solutions (Subsection 4.3.1).

4.2 Beginning the Test

At least ten fish per test vessel (replicate) must be used, with an equal number in each vessel. A test with five concentrations plus a control, with four replicates, requires at least 240 fish. The larvae should, if possible, represent three or more different spawnings. Each concentration including the control must start the test with the same number of replicates (at least three, Section 4.1).

Larvae must be ≤24 h old. There is some indication that variation in results may be caused by age differences within the 24-h collection period, perhaps because very young larvae with undeveloped swim bladders may be less tolerant of handling. It would be desirable, when feasible, to reduce that possible source of variation by selecting larvae with swim bladders already developed (Figure 3), and using them for the test. This would normally mean that the larvae would have been hatched for between ~7 and 24 h. Alternatively, more frequent inspection of the facilities used for hatching would allow selection of larvae that had been hatched for ≥7 and ≤24 h.

Since it is possible that larvae from a given spawning might be particularly sensitive or

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* It has been estimated that increasing the number of replicates from two to three in this test increased the amount of work by only 15%, but resulted in a major improvement in variation and sensitivity of the test. The addition of the third replicate was considered "worth the investment" (API, 1988). An increase from three to four replicates resulted in only a small additional decrease in variability; increased effort would not be warranted on that account, but use of four replicates is recommended here because of the infrequent but possible need in statistical analysis.

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[n] A comparison of mortality and swimming behaviour of fish in this control water versus the receiving-water control will distinguish any toxic responses that might be attributable to contaminants within the upstream water.

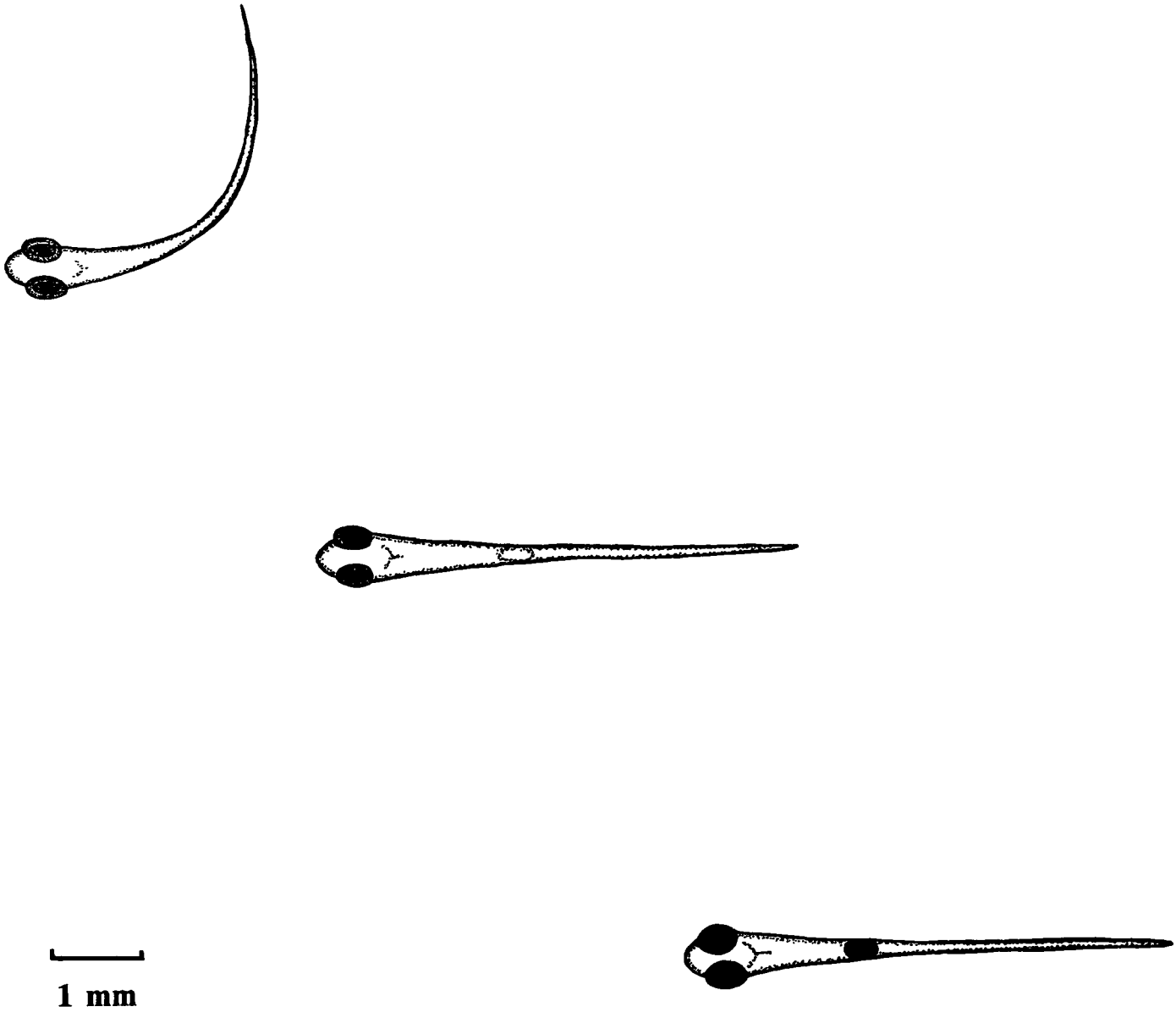

Figure 3 **Larvae of Fathead Minnows as They Appear if Viewed Dorsally**
(original drawings from specimans, by C.M. Neville and M.A. White)
On the upper left is a larva that has just hatched. The eyes are the most conspicuous feature. In the centre is a larva which hatched three or four hours earlier, and has not yet inflated its swim bladder. It may swim rapidly on the bottom of the container. On the lower right is a larvae with an inflated swim bladder, which might be ~7- to 24-h old. It can swim at any depth in the water.

particularly tolerant, an attempt must be made to achieve "homogeneity of the experimental units", i.e., to avoid any differences among vessels that are related to the spawning. There are two ways to achieve that. They are both valid and are suitable for the same statistical analyses of results (personal communication, Prof. J.J. Hubert, Dept of Mathematics and Statistics, Univ. of Guelph, Guelph, Ontario). In the first method, larvae from different parents or spawnings which have been held separately may be combined (pooled) before assigning larvae to vessels. In the second method, larvae from a given spawning may be divided evenly among all replicates of all concentrations, then larvae from other spawnings are similarly allotted evenly to all vessels, to make up the full number of 10. The second method requires more care and effort in culturing and handling. It should, however, reduce the "noise" of the variation between replicates at the same concentration and avoid the chance that exists in the first method, of getting high proportions of weak larvae or strong larvae in a particular vessel, assuming that such spawning-related variation exists. This latter method is recommended by Neville (1989).

With either of these methods, an attempt to achieve homogeneity must be made by assigning fish to vessels in the following manner. Larvae should be counted into a series of small beakers or plastic cups, introducing one, two, or three larvae at a time into each beaker in rotation, until the desired total numbers are attained in all. Fish appearing abnormal in any way should not be selected for the test. Fish should be moved by means of a large-bore pipette with rubber bulb, and any fish injured or possibly injured during transfer must be discarded. The amount of culture water carried over to the test vessels, with the fish in the pipette, must be minimal.

In addition to these procedures, there must be formal random assignment of the groups of ten or more larvae (those in the small beakers or cups) to particular concentrations and replicate numbers, or vice versa. The individual vessels must also be in randomized positions in the water bath or other temperature-control facility. Each test vessel must be clearly coded or labelled to identify the material and concentration being tested, and the date and time of starting. Temperature, dissolved oxygen, and pH levels in the vessels should be checked and adjusted, if required/permitted, to acceptable levels (Section 4.3, and Subsections 4.3.1 and 4.3.2) before introducing fish. As a check on test concentrations, it is recommended that conductivity be measured in each new preparation of test solution, before dispensing it to the test vessels.

4.3 Test Conditions

This is a 7-d test with replacement of solutions at 24-h intervals*. Fish are fed brine shrimp.

Daily mean temperature of all test solutions should be 25 ± 1°C with extreme fluctuations within the range 23 to 27°C, as determined by measurements in representative vessels at the beginning and end of 24-h periods, i.e., in the fresh test solution and the used solution just before it

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* Special situations (e.g., volatile or unstable chemicals in solution) require more frequent renewal of solutions, the use of flow-through tests, or modified duration of the test.

is changed, or just after it has been changed*.

The test is invalid if there is mortality or clearly atypical swimming in more than 20% of control fish**, if the average final weight of control fish does not attain 250 µg°, or if variability of replicates is too high (further detail in Section 4.5).

4.3.1 Dissolved Oxygen and Aeration

If (and only if) the measured dissolved oxygen is <40% or >100% of air saturation in one or more test solutions when they have been made up to start the test, all solutions should be aerated before the fish larvae are added ("pre-aeration"). Oil-free compressed air should be dispensed through a disposable glass pipette, with bubble size 1 to 3 mm, at a minimal rate for effective aeration of the particular vessel and volume of fluid being used. Duration of pre-aeration should be the lesser of 20 minutes and attaining 40% saturation in the highest test concentration (or 100% saturation, if supersaturation is evident) [p]. Any pre-aeration should be discontinued at 20 minutes and the test initiated, whether or not 40 to 100% saturation was achieved in all test solutions. Any pre-aeration must be reported (Section 8).

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* Although measurements in the old solution, after organisms have been moved to the new solution, are theoretically less relevant, there is a major advantage in using this approach, since no damage can be done to the fish larvae by the measuring device. The likelihood of damage may not be great for a thermometer, but is more likely for oxygen or pH probes which are moved around in the water.

** In a ten-laboratory round-robin comparison, average mortality in controls was 6%, and mortality was 20% or greater in only 16 of 270 individual control vessels (API, 1988).

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[o] Larval fathead minnows can be expected to average about 90 µg at the start of a test (API, 1988). Tests with good procedure should obtain a final average dry weight of 350 µg for control fish in soft water (hardness ≤50 mg/L), an average of 500 µg in water that is hard or moderately hard (hardness ≥130 mg/L), and proportional weights for the middle range of hardness. Measuring a statistically significant difference becomes more difficult with lower growth, and thus the test becomes less sensitive. On the basis of experience of Canadian and U.S. workers, an average dry weight of 250 µg or greater for control fish is a requirement for considering that the test is valid.

The API (1988) report on round-robin tests concluded that final larval weights varying from 120 to 650 µg did not influence the determination of endpoints. There was no obvious relationship between magnitude of NOEC determined at a laboratory and the amount of larval growth. Two of ten laboratories which did 12 to 14 tests each in the round-robin comparison did not attain an average control weight of 250 µg, five of them achieved an average over 400 µg, and three laboratories had the highest averages, in the 600 to 650 µg range. There was considerable within-laboratory variation about those averages. Despite the API conclusions, experience of Canadian workers indicates that poor growth of larvae (final weight <250 µg) is associated with poor discrimination in statistical tests and poor sensitivity of the test.

Experienced laboratories in U.S.A. get good growth, for example an average of 790 µg in 39 control groups with the lowest value 470 µg (Pickering, 1988), or 540 µg in various surface waters and 522 µg in soft water (Norberg and Mount, 1985). A round-robin test by ten laboratories near San Francisco yielded an average weight of 500 µg with standard deviation of 180 µg. The mean control mortality was 3% (Anderson and Norberg-King, 1991).

[p] Aeration can strip volatile chemicals from solution or can increase the rate of chemical oxidation and degradation to other substances. However, aeration of test solutions before fish exposure might be necessary due to the oxygen demand of the test material (e.g., oxygen depleted in the sample during storage). Aeration also assists in re-mixing the test solution. If it is necessary to aerate any test solution, *all* solutions are to be aerated in the manner stipulated in Subsection 4.3.1.

Dissolved oxygen must be recorded at the beginning of each 24-h period in representative concentrations of the freshly-prepared test solutions including the highest, which must again meet the requirements in the preceding paragraph. Measurements should also be made in representative concentrations at the end of 24-h periods, to establish the extent of oxygen depletion before the solutions are changed.*

Oxygen in the vessels should not fall below 40% of saturation (3.3 mg/L). If it does, the test becomes invalid as an assessment of the toxic quality, *per se*, of the material being tested. The test would still be a valid assessment of the total effect of the material (e.g., effluent) including its deoxygenating influence [q]. Potential problems with dissolved oxygen will be foretold by the initial measurements, and in such a case a running check on oxygen concentrations is required. The required use of oxygen-saturated control/dilution water and daily renewal of test solutions will, in most instances, keep dissolved oxygen above the levels that severely stress the larvae and have a major influence on test results. If the test material has a strong oxygen demand, more frequent renewal of test solutions might be required to maintain DO at ≥40% of saturation. If frequent renewal is not successful, and the objectives of the test require DO ≥40% saturation for a valid assessment of toxic qualities, then all vessels may be aerated with approximately 100 bubbles/min, using the tip of a pipette. Turbulence must be minimized in order to prevent stress to the fish. Aeration during testing must be reported (Section 8).

Alternatively, the objective of the test might require that oxygen demand be included as part of the measurement of total effects of the sample, in which case the daily renewal frequency would be retained, and no aeration would be used.

4.3.2 pH

The pH must be measured in the control, high, medium, and low concentrations at the beginning of the test, before fish are added. The pH should also be measured in representative vessels at the beginning and end of each 24-h period, i.e., in the fresh test solution and the used solution just before it is changed, or just after it has been changed.**

Toxicity tests should normally be carried out without adjustment of pH. However, if the sample of test material causes the pH of any test solution to be outside the range 6.5 to 8.5, and it is desired to assess toxic chemicals rather than the deleterious or

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* DO measurements may be made on a test solution after it has been removed from the test vessels by siphoning into a sample bottle, or other means that does not aerate it. This is allowed in order to avoid damage to the larvae (see footnote for temperature measurement in Section 4.3).

** See footnote to Section 4.3 concerning measurements on the old solution.

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q It should be realized that the lower limit of 40% saturation (3.3 mg/L) for dissolved oxygen in test solutions is an arbitrary one, and that oxygen levels above that value are also stressful to the fish. Growth of larval fathead minnows is reduced at 5 mg/L compared to growth at 7.2 mg/L (Brungs, 1971a). Any reduction below saturation, in fact, results in some metabolic loading of fish and decreases their performance (Doudoroff and Shumway , 1970). Thus at oxygen values above the limit of 3.3 mg/L, stress from low oxygen might be expected to interact with any stress from toxicants, and this will be measured as part of the effect of the sample, be it effluent or other test material. Such interaction has been accepted in this test procedure, as part of the impact being measured.

modifying effects of pH, then the pH of the solutions or sample should be adjusted before adding fish, or a second, pH-adjusted test should be conducted concurrently using a portion of the sample[r]. For this second test, the initial pH of the sample, or of each test solution[s] could, depending on objectives, be neutralized (adjusted to pH 7.0) or adjusted to within ± 0.5 pH units of that of the control/dilution water, before fish exposure. Another acceptable approach for this second test is to adjust each test solution, including the control, upwards to pH 6.5 to 7.0 (if test sample has/causes pH<6.5), or downwards to pH 8.0 to 8.5 (if sample has/causes pH >8.5). Solutions of hydrochloric acid (HCl) or sodium hydroxide (NaOH) at strengths ≤1 *N* should normally be used for all pH adjustments. Some situations (e.g., effluent samples with highly-buffered pH) might require higher strengths of acid or base.

In some circumstances it could be desired to carry out the most sensitive test possible for detecting toxic chemicals, rather than including pH as part of the total effect of a chemical, effluent, elutriate, or leachate. In such a case, depressing effects of low pH on growth and survival of larvae should be eliminated by raising pH of test solutions as necessary, to ≥6.8 or preferably ≥7.0 [h].

Abernethy and Westlake (1989) provide useful guidelines for adjusting pH. Aliquots of samples or test solutions receiving pH-adjustment[s] should be allowed to equilibrate after each incremental addition of acid or base. The amount of time required for equilibration will depend on the buffering capacity of the solution/sample. For effluent samples, a period of 30 to 60 minutes is recommended for pH adjustment (Abernethy and Westlake, 1989). Once the test is initiated, the pH of each test

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r The usual justification for not adjusting sample/solution pH is that pH might have a strong influence on the toxicity of a chemical, or substances in a wastewater. Thus, for the (generally) low concentrations of waste found in receiving water after dilution, any change from the natural pH, with concomitant modification of toxicity, should be accepted as part of the pollution "package". That leads to the rationale that the pH should not be adjusted in tests, and that is the requirement for the procedure given in this report, if test solutions are in the pH range 6.5 to 8.5.

Some chemicals and wastewaters, however, will create levels of pH that have appreciable direct sublethal or lethal effects at the high concentrations used in tests. That is especially true in monitoring or compliance tests with full-strength effluent. It seems unlikely that an investigator would be primarily interested in ascertaining whether extreme pH in full-strength effluent had a toxic effect on fish, because such a pH would be unrepresentative of what would prevail after even moderate dilution in receiving water. If pH *per se* were of primary interest, a toxicity test would not seem necessary, because the toxicity of extreme pH is well-documented, and any danger could be much more economically assessed by a simple physicochemical measurement. The investigator would usually wish to know if toxic substances were present in a wastewater, and determining that would require that any masking by toxic action of pH should be eliminated. That rationale leads to the use of pH-adjusted samples or test solutions, where appropriate. The rationale is exactly parallel to standardizing the temperature and dissolved oxygen in the toxicity tests, even if the wastewater itself were 90°C or had low (e.g., <2 mg/L) dissolved oxygen, either of which would have a rapid toxic effect in itself. Adjusting the pH before testing, or running a second pH-adjusted test, are options in the procedure described in this report, and the exact method for adjustment depends on the objectives of the test.

Investigators using the present test with fathead minnows should be aware that major effects on reproduction and larval survival have been found at about pH 6, with marginal effects apparent at pH 6.6. As discussed elsewhere[h], pH ≥6.8 and preferably ≥7.0 are recommended as a lower limit for eliminating effects of pH *per se*.

s Tests with chemicals or samples of effluent, leachate, or elutriate requiring pH adjustment usually require the separate adjustment of each test solution (including the control). Those with sample(s) of receiving water normally adjust an aliquot of the undiluted sample, before preparing the test concentrations.

solution is monitored (Section 4.4) but not adjusted.

If the purpose of the toxicity test is to gain an understanding of the nature of the toxicants in an effluent, elutriate, leachate, or receiving-water sample, pH adjustment is frequently used as one of a number of techniques (e.g., oxidation, filtration, air stripping, addition of chelating agent, etc.) for characterizing sample toxicity. Mount and Anderson-Carnahan (1988) list pH adjustment as one of nine "Toxicity Identification Evaluation" (TIE) techniques which, when performed with an acutely toxic aqueous sample, provide the investigator with a useful method for assessing the physical/chemical nature of the toxicant(s) and their susceptibility to detoxification.

4.3.3 Feeding

Fish are fed live brine shrimp nauplii (Subsection 2.3.9 and Appendix C) immediately after starting the test. The objective is to keep live brine shrimp available for the fish during the daylight hours, but not to have a large excess of these crustaceans in the test vessels. This is because brine shrimp will sorb some of the toxic material, and because they die after some time in the fresh water which could depress the dissolved oxygen level.

Groups of ten fish should be fed at least 1500 nauplii per day, and preferably 2250 nauplii/d. This should be in at least two feedings each day, starting in the early morning. Levels above that do not improve growth (Appendix C; Silberhorn, 1989). For more fish larvae per container, or fewer, numbers of nauplii should be appropriately adjusted.

There is no feeding during the final 12 hours of the test, in order to avoid weighing food in the gut of the fish.

4.3.4 Renewal of Test Solutions

This is a static-replacement test and the solutions are to be almost completely (≥80%) renewed at 24-h intervals after the start of the test. Siphoning or use of a pipette is the usual procedure. It is desirable to replace solutions in random order across the replicates within a concentration, particularly if the material being tested is difficult to keep mixed because some of the contents settle.

During renewal, dead brine shrimp and other detritus on the bottom of each chamber should first be removed. Then the remaining solution is drawn down to a height of 7 to 10 mm (sufficient to allow the fish to continue swimming). Depending on the shape of vessel, tilting might be required to achieve the 80% removal and the minimum height. New test solution is slowly added to make up the original total volume of test solution in each vessel. The entire procedure must be done cautiously to avoid any injury to the fish; working on a light-table assists in making the larval fish visible. In any case, the solution that is siphoned out or otherwise removed should go into a white tray, so that an inspection can be made for larvae that have been accidentally removed. Such fish are likely to be injured and should be discarded; the results of the test should be analyzed as if the discarded fish had not been present.

The siphoning procedure is much easier to accomplish without accidentally removing a fish, if the vessel is of the style with a screened sump. If containers with screened bottoms are used, siphoning of debris is done, then the container is quickly but gently removed from the vessel of old test solution

to a vessel that is ready with new solution. The momentary removal from liquid does no apparent harm to the larvae, if done delicately. Other techniques are allowed if the apparatus is made of nontoxic materials mentioned in Section 3.3, the required replacement of solution is obtained, and the control organisms show acceptable growth°. For example, a technique of "submerged pouring" of the larvae from a used screen-bottomed container to a new one was developed by Parrott (1988), allowing the fish to be held in containers that were clean at the start of each 24-h period.

4.4 Test Observations and Measurements

The mortality in each test vessel is to be deduced from a count of swimming larvae, at intervals of 24 hours from the start of exposure until the end of the test at 7.0 days of exposure. Loss of equilibrium or abnormal swimming behaviour should be recorded, along with the mortality.

Fish are considered dead when they fail to show any swimming activity, even when stimulated by a gentle jet of test solution from a wide-mouthed pipette. Any dead fish that are seen should be discarded. Often, dead larval fish will simply not be found upon inspection, because they decompose quickly or cannot be distinguished from debris on the bottom.

At the end of the 7.0-d exposure, living fish are counted, dried, and weighed. For *each vessel* of test solution, dry weight is determined for the fish as a group. Larvae may be transferred individually from the test chamber to a rinse of clean dilution water, to avoid including any debris in the weight. The rinse should be brief, no more than 5 min for the first larva to be transferred. After rinsing, the fish can be netted together and transferred to the weighing trays by means of fine forceps, making sure that no parts of the fish are broken off. Alternative techniques may be used with advantage [t].

Fish can be dried immediately in very small, tared and numbered aluminum weighing-boats, using a temperature of 100°C for at least 2 h and not more than 24 h. Upon removal from the oven, the boats are moved immediately to a desiccator, then the boats are individually and randomly removed from the desiccator, and weighed on a balance that measures consistently to 10 µg. The fish take up water vapour readily, so rapid weighing and standard timing among boats is necessary. (At the same time, care must be taken because rapid movement could blow any unattached larvae out of the weighing dish.) Trays should be removed in random order for weighing, and the first one weighed should be replaced in the desiccator and weighed at the end as a check on gain of water by the last trays weighed. The change should not be >5% [t]; if it is, redrying of the trays for <2 h and re-weighing might be carried out. A few weighing boats should be tared, dried, and weighed without fish, and results should conform to the laboratory's quality control

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t If they are demonstrated to be satisfactory, equivalent methods may be used for collecting the fish at the end of the test. One method successfully employed in a Canadian laboratory is to pour the contents of a test vessel onto a mesh of 1 mm pore size. Very hot water is immediately poured onto the mesh to kill and "fix" the fish, and they are then easy to handle with forceps. They must still be rinsed.

Alternative methods of weighing may also be used. For example, pairs of replicate weighing-boats or trays may be weighed together in a Latin square design so that each tray is weighed twice (trays 1 and 2, then 1 and 3, then 2 and 3). The weights of individual trays may then be estimated using algebra.

standards. Mean dry weight per fish is calculated. Alternatively, fish may be preserved in 70% ethanol at the end of the test, and within two weeks, rinsed in distilled water before drying and weighing in the same way as described for non-preserved fish.

Physicochemical measurements during the test, as described in Section 4.3, should include temperature, dissolved oxygen, and pH in representative test vessels at the beginning and end of each 24-h period. Conductivity should also be measured in the test solutions, at least at the start of the 24-h periods. Hardness of the control/dilution water and, as a minimum, the highest test concentration, should be measured and reported for the start of the test.

4.5 Test Endpoints and Calculations

There are two endpoints to the test, the first being adverse effect on growth of fish, measured as mean dry weights of the groups of fish from test vessels. The other endpoint is increased mortality. In both cases, the adverse effect is assessed by comparison with the controls. The most sensitive of the two effects is taken as the definitive indication of toxicity[u]. In addition, loss of equilibrium or clear-cut changes in swimming behaviour should be recorded since that could be used as a criterion of effect or of test validity.

At the end of the exposure, the number of fish alive and number dead are recorded for each replicate of the control and the various concentrations of the wastewater or chemical. (Fish that were accidentally killed or removed should be deducted from the total as if they had not been in the test.) The average dry weight per fish is calculated for the surviving fish in each vessel (i.e., in each replicate of each concentration and the control).

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u Length of fish should not be used as a criterion of effect because of increased body depth and weight among healthy fish in the last few days of the test, which is not adequately reflected in their length (Neville, 1989).

Although it might be thought that growth would be a much more sensitive criterion of effect than mortality, and a superior one because the interest is in sublethal effect, that is not necessarily so in this test. Death of the larval fish can apparently be one of the most sensitive effects in the life cycle of the fathead minnow. Woltering (1984) concluded from a major review of life-cycle and early-life-stage tests in fish that mortality of young stages was significant at the LOEC in more than half of the tests. In the ten-laboratory round-robin test, the relative sensitivity of the growth and mortality endpoints differed among the test materials. The two NOECs determined for growth and death were almost the same when testing pentachlorophenate and the effluent of one oil refinery. For potassium dichromate and another industrial effluent, weight was consistently more sensitive, while for another refinery effluent, mortality was sometimes the more sensitive response (API, 1988). If one compares only the results of the more successful tests in which final larval weight of controls exceeded 250 µg, the results of growth were much more sensitive than those for mortality; growth was most sensitive in 35 cases, mortality in 2, and equal results in 8 cases.

The authors of the API study comment that obtaining weights of fish increased the time of personnel by about 50% compared to observing mortality only, although that estimate seems unreasonably high. The price of a drying oven and a sensitive balance would also have to be added to the cost of doing the test if an industry were required to monitor its effluent on-site with this test, or chose to do so. API (1988) suggests that in cases where mortality was shown to be about equally sensitive as growth, for a particular effluent, it would be very cost-efficient to monitor that effluent using only mortality of larval fathead minnows.

Canadian experience indicates that weighing the larvae adds about half a day of work to the test. In most circumstances, the probability of increased sensitivity of the test would justify the extra investment of time and effort. If weights were not taken, reporting of results should clearly indicate that it was not done.

The test is invalid if mortality in the control water exceeds 20%, or if more than 20% of the fish in the control are moribund or display loss of equilibrium or clearly atypical swimming behaviour. The test is invalid if the average final weight of control fish does not attain 250 µg when the fish are dried and weighed immediately after the test. If fish are first preserved in 70% ethanol, the average weight of control fish must be 200 µg for the test to be considered valid. With reasonable procedures, it should not be difficult to attain average weights of 350 µg in soft water and 500 µg in hard water*°. If the Minimum Significant Difference for average weights of fish, provided by Dunnett's test (see below) is >25% of the mean weight of the controls, the variability of the results is such that validity and usefulness of the findings should be considered questionable.

Various endpoints can be calculated from the measurements at the end of the test, and the rationale and methods of calculation are discussed in detail by U.S. EPA (1989). No-observed-effect-concentrations and LOECs may be derived statistically by the hypothesis-testing approach, and this is recommended as a primary technique. Advice should be sought from a statistician in carrying out the analyses of results.

For weight of fish, the NOEC and LOEC are determined from the final dry weights of the groups of fish in each replicate of the control and the various concentrations of wastewater or chemical. If there is complete mortality in all replicates at a given concentration, that concentration is excluded from the analysis. For mortality, NOEC and LOEC are determined separately, in the same general manner. The lower set of NOEC/LOEC, for growth or mortality, is taken as the overall result of the test.

The statistical procedures to be followed for determining NOEC and LOEC are given in TOXSTAT^{v} (Gulley *et al.*, 1989). An up-to-date version of TOXSTAT can be obtained by contacting Environment Canada (see Appendix B). These statistics start with a check of normality and homogeneity of data, and provide suitable tests of significance for particular types of distribution. TOXSTAT also provides appropriate tests in cases where the numbers of replicates are unequal because of accidental loss or other cause. Usually, differences of each concentration from the control will be ascertained by Dunnett's test, a standard multiple-comparison test. Dunnett's test provides estimates of the Minimum Significant Difference, which is the magnitude of the difference in average weights or average mortality, that would have to exist between the control and a test concentration, before a significant effect could be concluded for that concentration. Dunnett's test is not a particularly powerful way of discriminating effects in toxicity tests since it ignores the information on the magnitude of the test concentrations (Masters *et al.*, 1991). Williams' test is also available in TOXSTAT and is designed to be sensitive to a response due to increasing concentration of toxicant (Gulley *et al.*, 1989). Williams' test is recommended as an alternative to Dunnett's test.

A geometric average of the NOEC and LOEC is often calculated for the convenience of having one number rather than two. Such a value may be used and

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* Control weights averaging greater than the required value of 250 µg, but less than the desirable values of 350 to 500 µg, might indicate that feeding or some other condition was less than favourable, although results of the test should still provide useful information (see footnote "o").

v The methods of TOXSTAT (Gulley *et al.*, 1989) are not detailed here because the instructions are best followed in the written description that accompanies the programs on computer diskette. Briefly, data are tested for normality by the *Shapiro-Wilks test*, and for homogeneity by *Bartlett's test*. If the data do not meet the requirements, it might be possible to transform them with logarithms or arc-sine to meet the requirements. It should be realized that the transformation, however, might reduce the sensitivity of the analysis and the ability of the toxicity test to detect differences.

If the data are regular or can be made so by suitable transformation, an analysis of variance is carried out. That is followed by *Dunnett's test*, a multiple-comparison test which assesses each concentration for significant difference from the control. It also estimates the Minimum Significant Difference (MSD) between groups, i.e., the difference in average weights or average mortality, that would have to exist between the control group and a test group before a significant difference could be concluded. If MSD of weight is 10 to 25% of the mean control dry weight, the test should be considered a reasonable assessment of sublethal toxicity. An MSD that is less than 10% of average control weight is likely to be in the potential range of variation of the control itself, and an MSD greater than 25% of control weight suggests less sensitivity than is desirable in detecting an effect (Neville, 1989, and personal communication).

Williams' test is recommended as a more powerful test to use instead of Dunnett's test (Section 4.5). At the time of writing, there do not seem to be guidelines for using values calculated by Williams' test to assess the general quality of the test and its results. If there are unequal numbers of replicates, the *Bonferroni t-test* is substituted for Dunnett's test.

If a set of data cannot meet the requirements for normality or homogeneity, and cannot be transformed to do so, there are non-parametric tests provided in TOXSTAT which may be substituted (*Steel's many-one rank test*, or the *Wilcoxon rank sum test* in the case of unequal replicates). Those nonparametric options may be used, and are powerful tools for data that are not normally distributed. It should be realized that the non-parametric tests are less powerful than parametric tests when used on normally-distributed data, and in that situation they might fail to detect real differences in effect, i.e., an underestimate of sublethal toxicity might result. It should also be remembered that four replicates are required to make use of the nonparametric methods.

Another option for analysis of results could be comparison of total biomass per vessel at the various concentrations. If initial numbers of fish were different in the vessels, there could be a comparison of the following statistic: (*total biomass surviving in the vessel*) divided by (*the number of fish that started the test in that vessel*). Reasons can be advanced that this is a more rational approach, and in particular, combining information on mortality and growth into one number is useful. The method will probably be more widely used in the future but has not found wide use at the time of writing, so cannot yet be considered a standard procedure. The method might be subject to further development, for example correction of experimental mortalities for any mortality in the control. (Individual fish that were accidentally killed by the investigator would not, of course, be included in the total number of fish considered to have started the experiment.)

A worthwhile supplemental or alternative measure of effect is estimation of the ICp or Inhibiting Concentration for a specified percentage effect. This statistic provides, by interpolation, a point estimate of a single concentration causing the specified percentage effect, rather than the pair of concentrations represented by NOEC and LOEC. A major disadvantage of the LOEC/NOEC pair of values is that variance and confidence limits cannot be calculated. Thus the ICp is useful for comparing toxicity of different wastewaters or chemicals, a comparison that cannot be approached in such a logical fashion, or with tests for significant differences, by means of the hypothesis-testing approach used for NOEC/LOEC. It should be remembered that an ICp, whether IC20, IC5, or IC1, is still, by definition, a concentration that causes the specified degree of harmful effect; it is not a no-effect concentration, technically speaking. That topic is well discussed in recent supplementary pages to the U.S. method (U.S. EPA, 1989).

An estimate of the IC20 or IC25 for weight, i.e., the concentration causing a 20% or a 25% reduction in dry weight of fish relative to the control, would often be a useful primary endpoint. The IC20 (or IC25) could be read from a graph which shows percentage reduction of weight against the logarithm of test concentration. Such a graph should be plotted to provide a visual assessment of the nature of the data, and to check any mathematical estimates. Computation of an ICp by the *linear interpolation* method may be done by the method in the fathead minnow section and Appendix J of U.S. EPA (1989). ICp and its 95% confidence limits can also be estimated by assumption-free linear interpolation using a computer program called BOOTSTRP (Norberg-King, 1988; U.S. EPA, 1989; Appendix B). The ICp procedure could also be used on total biomass as previously described, i.e., using data that incorporate both biomass and mortality. Wider use of the ICp is being seen at present.

reported, recognizing that it represents an arbitrary estimate of an effect-threshold that might lie anywhere in the range between the LOEC and NOEC. The calculated value of the geometric mean is governed by whatever concentrations the investigator happened to select for the test. No confidence limits can be estimated for the geometric mean, and that is also the case for NOEC and LOEC. The geometric mean of NOEC and LOEC is often called the *chronic value* in the United States, but that is misleading for this larval exposure which represents 1% or 2% of a fathead minnow's lifetime and is therefore not chronic. The mean is sometimes called a *subchronic value*. That term might be used, or "*TEC*" signifying *threshold-effect concentration* might be an appropriate name for the calculated value. That use of "threshold" is in the dictionary sense of "point at which an effect begins to be produced".

As an alternative or additional primary technique which is now seeing greater use, the ICp *(inhibiting concentration for a specified percentage effect)* may be calculated as a point-estimate of the concentration causing a certain degree of effect. The percentage is selected by the investigator, and is customarily 20% or 25% reduction in growth or increase in mortality compared to the control. The ICp is a useful measure of effect, often more sensitive and more desirable than NOEC/LOEC values determined by hypothesis testing (Suter *et al.*, 1987). In particular, confidence limits can be calculated, allowing statistical comparisons with other such values. Such an analysis could begin with a plot of percentage reduction of growth against the logarithm of test concentration, with IC20 or IC25 read off. The graph would also serve as a check against results from mathematical computations. A straightforward *linear interpolation* method provides a mathematical estimate of the ICp (U.S. EPA, 1989, Appendix J). The ICp and confidence limits may be estimated by a "bootstrap" method on computer (Norberg-King, 1988; U.S. EPA, 1989) [v]. An up-to-date version of BOOTSTRP can be obtained by contacting Environment Canada (see Appendix B).

An LC_{50} might sometimes be calculated in multi-concentration tests, and would be a desirable point-estimate of larval mortality. The LC_{50} would be for a defined period, normally for the seven days of exposure. The range of test concentrations might be extended upwards to ensure greater than 50% mortality in at least one concentration, so that the LC_{50} could be estimated. There are dependable methods available for calculating the LC_{50} and its 95% confidence limits, methods that are generally familiar to aquatic toxicologists (Appendix E).

Another approach analyzes the total biomass per vessel, and makes use of both the weight of larvae and the number surviving [v]. This is a powerful approach and is recommended.

In a single-concentration test, a t-test is normally the appropriate method of comparing the data from the test concentration with those of the control, and the procedure for a t-test can be taken from any statistics textbook. An effect of the test material is accepted if weights are significantly lower, or mortality is significantly higher, than the same statistics for the control. Requirements for homogeneity of variance and normality must be satisfied (Appendix H of U.S. EPA, 1989; Gulley *et al.*, 1989) before using the standard t-test. If the data do not satisfy the requirements, a nonparametric test could be selected with advice from a statistician; no particular test appears to have become standard practice as yet.

In some cases, the test groups might not represent various concentrations of a single sample of wastewater or chemical, but rather a set of different samples, such as full-strength effluents from different industries, or samples of surface waters from different places. It might be desired to test not only whether each sample is different from the control, but also whether the samples are different from each other. That can be done using one option in the statistical program TOXSTAT (Tukey's test[v]). Such sets of tests should report the results of each sample tested, not as NOEC/LOEC, but as the mortality (or weight) as a percentage of the control(s), and whether that number was significantly different from the control(s).

4.6 Reference Toxicant

The routine use of a reference toxicant or toxicants is necessary to assess, under standardized test conditions, the relative sensitivity of the groups of fish that are used, and the precision and reliability of data produced by the laboratory for that/those reference toxicants (Environment Canada, 1990c). Sensitivity of fish to the reference toxicant(s) should be evaluated at least once each month that larvae from the culture are used in toxicity tests.

Criteria used in recommending appropriate reference toxicants for this test include:

- chemical readily available in pure form;
- stable (long) shelf life of chemical;
- highly soluble in water;
- stable in aqueous solution;
- minimal hazard posed to user;
- easily analyzed with precision;
- good dose-response curve for fathead minnows;
- known influence of pH on toxicity of chemical to test organism; and
- known influence of water hardness on toxicity of chemical to fathead minnows.

Reagent-grade sodium chloride, phenol, and/or zinc (prepared using zinc sulphate) are recommended for use as the reference toxicants for this test. Fish sensitivity should be evaluated by standard tests following the methods given in this document, to determine the NOEC/LOEC and/or ICp for one or more of these chemicals. The tests should use the control/dilution water that is customary at the laboratory, or reconstituted water if a greater degree of standardization is desired[w].

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[w] Because the pH, hardness, and other characteristics of the dilution water can markedly influence the toxicity of the test material, use of a standard reconstituted water provides results that can be compared in a meaningful way with results from other laboratories.

Soft reconstituted water is recommended for this purpose. This water is prepared by adding the following quantities of reagent-grade salts to carbon-filtered, deionized water, or glass-distilled water (ASTM, 1980):

	salt	mg/L
Sodium bicarbonate	$NaHCO_3$	48
Calcium sulphate	$CaSO_4 \cdot 2H_2O$	30
Magnesium sulphate	$MgSO_4$	30
Potassium chloride	KCl	2

The reconstituted water should be aged several days (U.S. EPA, 1985) and intensely aerated before use. It can be expected to have a total hardness of 40 to 48 mg/L and pH of 7.4 ± 0.2.

Test conditions and procedures for tests with reference toxicants are to be consistent and as described in this document.

A warning chart (Environment Canada, 1990c) should be prepared and updated for each reference toxicant used. Successive NOECs or ICps are plotted on this chart and examined to determine whether the results are within ± 2 SD of values obtained in previous tests. The geometric mean NOEC or ICp, together with its upper and lower warning limits (± 2 SD calculated on a geometric [logarithmic] basis)*, is recalculated with each successive NOEC or ICp until the statistics stabilize (U.S. EPA, 1989; Environment Canada, 1990c).

If a particular NOEC or ICp falls outside the warning limits, the sensitivity of the fish and the test system are suspect. Inasmuch as this might occur 5% of the time due to chance alone, an outlying value does not necessarily mean that the sensitivity of the population of fish or the precision of the toxicity data produced by the laboratory are in question. Rather, it provides a warning that this might be the case. A check of all holding and test conditions is required at this time.

Use of warning limits does not necessarily indicate that a laboratory is generating consistent results. A laboratory that produced extremely variable data for a reference toxicant would have wide warning limits; a new data point could be within the warning limits but still represent undesirable variation in results obtained in tests. A coefficient of variation of 20% or 30% is tentatively suggested as a limit by Environment Canada (1990c). That seems a reasonable range since round-robin tests in the San Francisco area showed a coefficient of variation between laboratories of 22% when calculated on a logarithmic basis (C.V. = 31% when calculated on an arithmetic basis; Anderson and Norberg-King, 1991). However, establishing a limit for allowable variation of results for testing reference toxicants would require more data on the reproducibility that can be achieved in Canadian laboratories for the seven-day test with fathead minnows.

Stock solutions of phenol should be made up on the day of use. Zinc sulphate (usually $ZnSO_4 \cdot 7H_2O$, molecular weight 4.398 times that of zinc) should be used for preparing stock solutions of zinc, which should be acidic (pH 3 to 4). Acidic zinc solutions may be used when prepared, or stored in the dark at 4 ± 2°C for several weeks before use. Concentrations of zinc should be expressed as mg Zn^{++}/L. Concentrations of sodium chloride should be expressed as the weight of the total salt (NaCl) in the water (g/L).

Concentrations of reference toxicant in all stock solutions should be measured chemically by appropriate methods (e.g., APHA *et al.*, 1989). Upon preparation of the test solutions, aliquots should be taken from at least the control, low, middle, and high concentrations, and analyzed directly or stored for future analysis should the NOEC or ICp be atypical (outside warning limits). If stored, sample aliquots must be held in the dark at 4 ± 2°C. Both zinc and phenol solutions should be preserved before storage (APHA *et al.*, 1989). Stored aliquots requiring chemical measurement should be analyzed promptly upon completion of the toxicity test. It is desirable to measure concentrations in the same solutions at the

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* If the NOEC or ICps fail to show a lognormal distribution, an arithmetic mean and SD might prove more suitable.

end of the test, after completing biological observations. Calculations of NOEC or ICp should be based on the geometric mean measured concentrations if they are appreciably (i.e., ≥20%) different from nominal ones and if the accuracy of the chemical analyses is satisfactory.

4.7 Legal Considerations

Care must be taken to ensure that samples collected and tested with a view to prosecution will be admissible in court. For this purpose, legal samples must be: representative of the material being sampled; uncontaminated by foreign substances; identifiable as to date, time, and location of origin; clearly documented as to the chain of continuity; and analyzed as soon as possible after collection. Persons responsible for conducting the test and reporting the findings must maintain continuity of evidence for court proceedings (McCaffrey, 1979), and ensure the integrity of the test results.

Section 5

Specific Procedures for Testing Chemicals

This section gives particular instructions for testing chemicals, in addition to the procedures in Section 4. For testing chemicals, a multiple-concentration test is usually performed, to determine the NOEC/LOEC and/or ICp. Three replicates, the minimum required for statistical analysis of results, might also be required under regulations for registering a pesticide or similar category of chemical.

5.1 Properties, Labelling, and Storage of Sample

Information should be obtained on the properties of the chemical to be tested, including water solubility, vapour pressure, chemical stability, dissociation constants, and biodegradability. Datasheets on safety aspects of the material should be consulted, if available. Where aqueous solubility is in doubt or problematic, acceptable procedures used previously for preparing aqueous solutions of the chemical should be obtained and reported. Other available information such as structural formula, degree of purity, nature and percentage of significant impurities, presence and amounts of additives, and n-octanol:water partition coefficient should be obtained and recorded[x]. An acceptable analytical method should also be known for the chemical in water at concentrations intended for the test, together with data on precision and accuracy.

Chemical containers must be sealed and coded or labelled (e.g., chemical name, supplier, date received) upon receipt. Storage conditions (e.g., temperature, protection from light) are frequently dictated by the nature of the chemical. Standard operating procedures for chemical handling and storage should be followed.

5.2 Preparing Test Solutions

Test solutions of the chemical are usually prepared by adding aliquots of a stock solution made up in control/dilution water. Alternatively, for strong solutions or large volumes, weighed (analytical balance) quantities of chemical may be added to control/dilution water to give the nominal strengths for testing. If stock solutions are used, the concentration and stability of the test chemical in the solution should be determined before the test. Stock solutions subject to photolysis should be shielded from light, and unstable solutions must be prepared as frequently as necessary to maintain concentrations for each renewal of test solutions.

For chemicals that do not dissolve readily in water, stock solutions may be prepared using the generator-column technique (Billington *et al.*, 1988; Shiu *et al.*, 1988) or, less desirably, by ultrasonic dispersion. Ultrasonic dispersion can produce droplets that differ in size and uniformity, some of which might migrate towards the surface of

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[x] Knowledge of the properties of the chemical will assist in determining any special precautions and requirements necessary while handling and testing it (e.g., testing in a well-ventilated facility, need for solvent, etc.). Information regarding chemical solubility and stability in fresh water will also be useful in interpreting test results.

the liquid, or vary in biological availability creating variations in toxicity. Organic solvents, emulsifiers, or dispersants should not be used to increase chemical solubility except in instances where they might be formulated with the test chemical for its normal commercial purposes. If used, an additional control solution should be prepared containing the same concentration of solubilizing agent as in the most concentrated solution of the test chemical. Such agents should be used sparingly, and should not exceed 0.1 mL/L in any test solution. If solvents are used, the following are preferred (U.S. EPA, 1985): dimethyl formamide, triethylene glycol, methanol, ethanol, and acetone.

5.3 Control/Dilution Water

Control/dilution water may be reconstituted water, the freshwater source in which the adults were cultured and the larvae hatched (natural groundwater, surface water, or dechlorinated municipal water as a last choice)[e], or a particular sample of receiving water if there is special interest in a local situation. The choice of control/dilution water depends upon the intent of the test.

If a high degree of standardization is required (e.g., the measured toxicity of a chemical is to be assessed relative to values derived elsewhere, for this and/or other chemicals), soft reconstituted water (hardness 40 to 48 mg/L as $CaCO_3$, pH 7.2 to 7.5) should be prepared and used for all dilutions and as the control water[w] (U.S. EPA, 1985).

If the toxic effect of a chemical on a particular receiving water is to be appraised, sample(s) of the receiving water could be taken from a place that was isolated from influences of the chemical, and used as the control/dilution water[y,z,aa]. Examples of such situations include appraisals of the toxic effect of chemical spills (real or potential) or intentional chemical applications (e.g., spraying of a pesticide) on a particular waterbody. The laboratory supply of natural water may also be used for this purpose, especially where the collection and use of receiving water is impractical. Normal laboratory water in which fish have

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y Contaminants already in the receiving water might add toxicity to that of the chemical or wastewater being tested. In such cases, uncontaminated dilution water (reconstituted, natural, or dechlorinated municipal) would give a more accurate estimate of the individual toxicity of the spill or spray, but not necessarily of the total effect on the site of interest.

If the intent of the test is to determine the effect of a specific chemical or wastewater on a specific receiving water, it does not matter if that receiving water modifies sample toxicity by the presence of additional toxicants, or conversely by the presence of substances that reduce toxic effects, such as humic acids. In the case of toxicity added by the receiving water, it would be appropriate to include in the test, as a minimum, a second control of culture water and, as a maximum, another series of concentrations using culture water as diluent.

z While it would be desirable to acclimate the breeding fish, and hold the embryos in the receiving water before using the larvae in a test with that water used for dilution and control, that is seldom feasible because of the need to transport large volumes of water. If tests were carried out near the site of interest, it might be feasible to use receiving water in the breeding aquaria for at least five days before embryos were selected, and to hold the embryos in receiving water until the larvae had hatched.

aa An alternative (compromise) to using receiving water as dilution and control water is to adjust the pH and hardness of the laboratory water supply (or reconstituted water) to that of the receiving water. Depending on the situation, the adjustment might be to seasonal means, or to values measured in the receiving water at a particular time. Adjustments may be made by methods mentioned in Subsection 2.3.4, including addition of appropriate quantities and ratio of reagent-grade salts (ASTM, 1980; also given in Table 2 of Environment Canada, 1990b).

been cultured is also appropriate for use in other instances (e.g., preliminary or intra-laboratory assessment of chemical toxicity).

5.4 *Test Observations and Measurements*

In addition to the observations on toxicity described in Section 4.4, there are certain additional observations and measurements to be made during tests with chemicals.

During preparation of solutions and at each of the prescribed observation periods during the test, each solution should be examined for evidence of chemical presence and change (e.g., odour, colour, opacity, precipitation, or flocculation). Any observations should be recorded.

It is desirable and recommended that test solutions be analyzed to determine the concentrations of chemicals to which fish are exposed[bb]. If chemicals are to be measured, sample aliquots should be taken from at least the high, medium, and low test concentrations, and the control(s). As a minimum, separate analyses should be performed with samples taken at the beginning and end of the renewal periods on the first and last days of the test. These should be preserved, stored and analyzed according to best proven methodologies available for determining the concentration of the particular chemical in aqueous solution.

If chemical measurements indicate that concentrations declined by more than 20% during the test, the toxicity of the chemical should be re-evaluated by a test in which solutions are renewed more frequently than once a day. If necessary, a flow-through test could be considered although it requires special design to accommodate the small larvae (McKim, 1985).

Toxicity results for any tests in which concentrations are measured should be calculated and expressed in terms of those measured concentrations, unless there is good reason to believe that the chemical measurements are not accurate. In making the calculations, each test solution should be characterized by the geometric average measured concentration to which fish were exposed.

5.5 *Test Endpoints and Calculations*

The endpoint for tests performed with chemicals will usually be the NOEC/LOEC and/or ICp for growth and mortality, i.e., one or more of the primary endpoints described in Section 4.5.

If a solvent control is used, the test is rendered invalid if mortality in this control (or in the untreated control water) exceeds 20%. The test is also invalid if >20% of the fish in either control display atypical/stressed behaviour such as erratic swimming or loss of equilibrium

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bb Such analyses need not be undertaken in all instances, due to analytical limitations, cost, or previous technical data indicating chemical stability in solution under conditions similar to those in the test.

Chemical analyses are particularly advisable if (U.S. EPA, 1985): the test solutions are aerated; the test material is volatile, insoluble, or precipitates out of solution; the test chemical is known to sorb to the material(s) from which the test vessels are constructed; or a flow-through system is used. Some situations (e.g., testing of pesticides for purposes of registration) might require the measurement of chemical concentrations in test solutions.

(Sections 4.3, 4.4, and 4.5). If Dunnett's test (Section 4.5 [v]) estimates that the Minimum Significant Difference for the weights of fish is >25% of the mean control weight, the validity and usefulness of the results should be considered questionable.

Section 6

Specific Procedures for Testing Effluent, Elutriate, and Leachate Samples

This section gives particular instructions for testing samples of effluent, elutriate, and leachate, in addition to the procedures listed in Section 4.

6.1 Sample Collection, Labelling, Transport, and Storage

Containers for transportation and storage of samples of effluent, leachate, or elutriate must be made of nontoxic material. Glass or Teflon™-coated containers are preferred as they are inert and reduce sorption of chemicals. Polyethylene or polypropylene containers manufactured for transporting drinking water are less desirable but may also be used. The containers must either be new or thoroughly cleaned and rinsed with uncontaminated water. They should also be rinsed with the sample to be collected. Containers should be filled to minimize any remaining air space.

Most tests with effluent, elutriate, or leachate will be performed "off-site" in a controlled laboratory facility. Effluents or leachates for off-site testing should be collected on three occasions at intervals of two to three days. The first sampling should provide fresh material for the first and second days of the test, the second sampling for the third and fourth days, and the third sample for the fifth, sixth, and seventh test days. For elutriates, fresh aliquots of sample should also be prepared and delivered to the laboratory for use on the same schedule, if possible. Storage of elutriate samples for seven days before use is undesirable because the sample might not be stable for such a period. If effluents or leachates are tested in on-site laboratories, samples should be collected daily and used within 24 h for each daily replacement of test solutions (U.S. EPA, 1989).

A 4-L sample is adequate for an off-site multiple-concentration test (e.g., concentrations of 100, 50, 25, 12.5, 6.3, 3.2, 1.6%) and for associated routine sample analysis. Smaller amounts are required for single-concentration tests (Section 4.5). Upon collection, each sample container must be filled, sealed, and labelled or coded. Labelling should include at least sample type, source, date and time of collection, and name of sampler(s). Unlabelled or uncoded containers arriving at the laboratory should not be tested. Nor should samples arriving in partially-filled containers be routinely tested, because volatile toxicants escape into the air space. However, if it is known that volatility is not a factor, such samples might be tested at the discretion of the investigator.

Testing of effluent and leachate samples should commence as soon as possible after collection. Whenever possible, testing should begin within 24 h, and must commence no later than 72 h after sampling. Samples collected for extraction and subsequent testing of the elutriate should be tested as soon as possible and no later than ten days following their receipt. Elutriate tests should commence within 72 h of preparation or as specified in a regulation or protocol.

All samples of effluent or leachate should be kept cool (1 to 7°C, preferably 4 ± 2°C) throughout their period of transport and storage. Upon collection, warm (>7°C) samples should be cooled to 1 to 7°C with ice or frozen gel packs. As necessary, gel packs or other means of refrigeration should be used to assure that sample temperature remains within 1 to 7°C during transit. Samples must not freeze during transport.

Upon arrival at the laboratory, an aliquot of effluent or leachate required at that time may be adjusted immediately or overnight to 25°C, and used in the test. The remaining portion(s) of sample required for subsequent solution renewals should be stored in darkness in sealed containers at 1 to 7°C, and preferably at 4 ± 2°C.

Temperature conditions should also be as indicated for transportation and storage of elutriates, as well as for samples intended for aqueous extraction and subsequent testing of elutriate, unless otherwise specified.

6.2 Preparing Test Solutions

Samples in the collection containers must be agitated thoroughly just before pouring to ensure the re-suspension of settleable solids. Subsamples (i.e., a sample divided between two or more containers) must be mixed together to ensure their homogeneity. If further sample storage is required, the composited sample (or a portion thereof) should be returned to the subsample containers and stored (Section 6.1) until used.

Any samples that might contain small organisms that could attack larval fathead minnows or compete with them should be filtered through a 60-µm plankton net before use (U.S. EPA, 1989). Such filtration could remove suspended solids that are characteristic of the sample and might otherwise contribute part of the toxicity or modify the toxicity. If there is such a concern, a second and concurrent test should be conducted using an unfiltered portion of the sample.

6.3 Control/Dilution Water

Tests with samples of effluent or leachate, intended to assess compliance with regulations, should use as the control/dilution water, either the laboratory water normally supplied to the fish, or a sample of the receiving water. Because results could be different for the two sources of water, the objectives of the test must be decided before a choice is made. Shipping difficulties and costs should also be considered, as the use of receiving water for dilutions and as control water greatly increases the volume of liquid to be shipped.

The use of receiving water as the control/dilution water might be desirable in certain instances if site-specific information is required for the potential toxic effect of an effluent, leachate, or elutriate on a particular receiving water[y,z,aa]. An important example of such a situation would be testing for sublethal effect at the edge of a mixing zone, under site-specific regulatory requirements. Conditions for the collection, transport, and storage of such receiving-water samples should be as described in Section 6.1. Surface water should be filtered to remove organisms, as described in Section 6.2.

If a sample of upstream receiving water is to be used as control/dilution water, a separate control solution should be prepared using the laboratory water supplied to the fish. Growth, mortality, and swimming behaviour of fish (Section 4.5) in the laboratory control

water should be compared to that in the sample of receiving water.

Tests requiring a high degree of standardization may be undertaken using reconstituted water as the control/dilution water[w]. This would be appropriate if it were desirable to minimize any modifying influence due to (differing) dilution-water chemistry. Such situations might include studies intended to interrelate toxicity data for various effluent, leachate, or elutriate types and sources, derived from a number of test facilities or from a single facility where water quality was variable.

6.4 Test Conditions

Samples of effluent, leachate, or elutriate are normally not filtered or agitated during the test. However, the presence of high concentrations of suspended inorganic or organic solids in a sample could be particularly stressful to larval fish, and can be acutely lethal, even to juvenile fish if present in sufficiently high strengths (e.g., ≥2000 mg/L, Noggle, 1978; McLeay *et al.*, 1987; Servizi *et al.*, 1987; Hall and Hall, 1989). High concentrations of biological solids in certain types of treated effluent might also contribute to sample toxicity because of ammonia and/or nitrite production (Servizi and Gordon, 1986). An additional test should be conducted if there is concern about a contribution to toxicity by elevated concentrations of suspended or settleable solids in samples of effluent, elutriate, or leachate, and if the intent of the study is to quantify the degree to which sample solids contribute to toxicity. The second test should use a portion of the sample, treated by filtering or decanting to remove solids, but procedures should be otherwise identical.

6.5 Test Observations and Measurements

Mortality and swimming behaviour at 24-h intervals and dry weight at the end of the 7.0-d test should be observed as in Section 4.4.

Colour, turbidity, odour, and homogeneity (i.e., presence of floatable material or settleable solids) of the sample of effluent, leachate, or elutriate should be observed at the time of preparing test solutions. Precipitation, flocculation, colour change, odour, or other reactions upon dilution with water should be recorded, as should any changes in appearance of solutions during the test (e.g., foaming, settling, flocculation, increase or decrease in turbidity, colour change).

For tests with highly coloured or opaque solutions, or for samples producing foam in the test vessel, tests should use the screen-bottomed vessels mentioned in Section 3.3 and Subsection 4.3.4. Fish should be inspected by raising the vessel in its container of test solution until they can be seen. If necessary, the vessel could be moved briefly to a container of clear dilution water while observations were made on mortality and aberrant swimming behaviour. Experience indicates that the brief period of transfer between liquids and of immersion in a "clean" liquid does not damage the fish to any degree or noticeably affect the results of the toxicity test (Parrott, 1988).

For effluent samples with appreciable solids content, it is desirable to measure total suspended and settleable solids (APHA *et al.*, 1989) upon receipt, as part of the overall description of the effluent, and as sample characteristics that might influence the results of the toxicity test.

6.6 Test Endpoints and Calculations

Tests for monitoring and compliance with regulatory requirements should normally include, as a minimum, three or more undiluted portions of the sample, and three or more replicate control solutions. Depending on specified regulatory requirements, tests for regulatory compliance might use a single concentration (100% wastewater unless otherwise specified), might determine the NOEC/LOEC and/or ICp, or might determine the LC_{50} at seven days or other exposure time, if mortality is severe (see Section 4.5).

Toxicity tests conducted for other purposes (e.g., determination of in-plant sources of toxicity, treatment effectiveness, effects of process changes on toxicity) might, depending on the study objectives, be single-concentration tests (100% or an appropriate dilution, plus a control), or multiple-concentration tests. Single-concentration tests are often cost-effective for determining the presence or absence of measurable toxicity or as a method for screening a large number of samples for relative toxicity. Endpoints for these tests would again depend on the objectives of the undertaking, but could include arbitrary "pass" or "fail" ratings, or percentage mortality of fish at a suitable time period such as seven days. Items in Section 4.5 provide instructions that are relevant here, on statistical analysis and reporting of results from a set of tests on different samples, each tested at only one concentration.

Section 7

Specific Procedures for Testing Receiving-water Samples

Instructions for testing samples of receiving waters, additional to those provided in Section 4, are given here.

7.1 Sample Collection, Labelling, Transport, and Storage

Procedures for the labelling, transportation, and storage of samples should be as described in Section 6.1. Testing of samples should commence as soon as possible after collection, preferably within 24 h, and no later than 72 h after sampling.

7.2 Preparing Test Solutions

Samples in the collection containers should be agitated before pouring to ensure their homogeneity. Compositing of sub-samples should be as described in Section 6.2.

Samples that might contain predators or competitors of larval fathead minnows should be filtered through a 60-μm plankton net before use, and a second unfiltered test could be run if there is concern about changes in toxicity, as described in Section 6.2.

7.3 Control/Dilution Water

For receiving-water samples collected in the vicinity of a wastewater discharge, chemical spill or other point-source of possible contamination, "upstream" water may be sampled concurrently and used as control water and diluent for the downstream samples[z,aa]. This control/dilution water should be collected as close as possible to the contaminant source(s) of concern, but upstream of the zone of influence or outside it. Such surface water should be filtered to remove organisms, as described in Section 6.2.

If "upstream" water is used as control/ dilution water, a separate control solution should be prepared using the laboratory water that is normally supplied to the fish. Test conditions and procedures for preparing and evaluating each control solution should be identical, and as described in Sections 4.1 and 5.3.

Logistic constraints, expected toxic effects, or other site-specific practicalities might prevent or rule against the use of upstream water as the control/dilution water. In such cases, the laboratory water supply used for rearing fish should be used as control water and for all dilutions. It could be adjusted to partially simulate upstream water[aa].

7.4 Test Observations and Measurements

Observations such as sample and solution colour, turbidity, foaming, and precipitation, should be made as described in Section 6.5, both during preparation of solutions and subsequently during the tests. These are in addition to the primary observations on fish described in Section 4.4.

7.5 Test Endpoints and Calculations

Endpoints for tests with samples of receiving water should be consistent with the options and approaches identified in Sections 4.5

and 6.6. Endpoints would normally be the same items mentioned in Section 4.5, i.e., mortality, growth, and swimming behaviour of larval fish.

Tests for monitoring and compliance purposes should normally include, as a minimum, three or more undiluted portions of the sample, and three or more replicate control solutions. If toxicity of receiving-water samples is likely, and information is desired concerning the degree of dilution necessary to permit normal growth and development of larval fish, a full test to determine NOEC/LOEC and/or ICp should be conducted as outlined in Section 4, with one or more undiluted (100% sample) concentrations as the highest concentration in the series tested.

Certain sets of tests might use a series of samples such as surface waters from a number of locations, each tested at full strength only. Statistical testing and reporting of results for such tests should follow the procedures outlined in Section 4.5.

Section 8

Reporting Requirements

The test report should describe the materials and methods used, as well as the results. A reader should be able to establish from the report whether the conditions and procedures rendered the results acceptable for the use intended.

Procedures and conditions that are common to a series of ongoing tests (e.g., routine toxicity tests for monitoring or compliance purposes), and consistent with specifications in this document, may be referred to by citation or by attachment of a general report that outlines standard laboratory practice. Where choices exist, the approach selected should be specified. The general report should convey the procedural information included in Sections 8.2 to 8.6. An individual test report giving the findings should contain the information indicated in Sections 8.1 and 8.7. Specific monitoring programs might require other selected items in the test report (e.g., procedures and results for tests requiring pH adjustment or modified aeration/oxygenation). Other details pertinent to the conduct and findings of the test, that are not conveyed by the reports, should be kept on file by the laboratory, so that the appropriate information can be provided if an audit of the test is required.

8.1 Test Material

- sample type, source, and description (chemical, effluent, elutriate, leachate, or receiving water; sampling location and method; specifics regarding nature, appearance and properties, volume and/or weight);
- information on labelling or coding of the test material;
- details on manner of sample collection, transport and storage (e.g., batch, grab or composite sample; description of container; temperature of sample upon receipt and during storage);
- identification of person(s) collecting and/or providing the sample; and
- dates and times for sample collection, receipt at test facility, start and end of definitive test.

8.2 Test Organisms

- species and source;
- description of culturing and breeding conditions (facilities, lighting, water source and quality, water pre-treatment, water exchange rate and method, density of fish in culture and breeding aquaria, temperatures in those aquaria, food type, ration, frequency of feeding, and disease incidence and treatment);
- weekly percentage of mortalities among the fish being grown to maturity and the breeding population; and
- for those embryos being cultured, approximate percentage hatching success; for larvae being reared, approximate mortality from hatching to 30 days of age.

8.3 Test Facilities and Apparatus

- name and address of test laboratory;
- name of person(s) performing the test;
- description of systems for regulating light and temperature within test facility; and
- description of test vessels (size, shape, type of material).

8.4 Control/Dilution Water

- type(s) and source(s) of water used as control and dilution water;
- type and quantity of any chemical(s) added to control or dilution water;
- sampling and storage details if the dilution water was "upstream" receiving water;
- water pre-treatment (temperature adjustment, de-gassing, aeration rates and duration, etc.); and
- measured water-quality variables (Subsection 2.3.4) before and/or at time of commencement of toxicity test.

8.5 Test Method

- brief mention of method used if standard (e.g., as per this document);
- design and description if specialized procedure (e.g., renewal of test solutions at intervals other than daily, or continuous replacement of solutions) or modification of standard method;
- procedure used in preparing stock and/or test solutions of chemicals;
- any chemical analyses of test solutions and reference to analytical procedure(s) used;
- use of preliminary or range-finding test; and
- frequency and type of observations made during test.

8.6 Test Conditions

- number, concentration, volume, and depth of test solutions including controls;
- number of organisms per solution;
- photoperiod, light source, and intensity at surface of test solutions;
- statement concerning aeration (if any, give rate, duration, manner of application) of test solutions before and during exposure of fish;
- description of any test solutions receiving pH adjustment or filtration, including procedure;
- any chemical measurements on test solutions (e.g., chemical concentration, suspended solids content);
- temperature, pH, dissolved oxygen (mg/L and % saturation), and conductivity as measured/monitored in each test solution; total hardness of control/dilution water and the highest test concentration at the start of the test; and
- conditions and procedures for measuring the NOEC/LOEC and/or ICp for the reference toxicant(s).

8.7 Test Results

- appearance of test solutions and changes noted during test;
- swimming behaviour and number and percentage of mortalities in each test solution (including control) as noted during each observation period and at the end of the test; number and percentage of control fish strongly showing atypical swimming behaviour;
- results for range-finding test (if conducted);
- the NOEC/LOEC and/or ICp for growth of larvae and for mortality; Minimum Significant Difference in average weights, and weight of control fish; the statistical test(s) used, and any transformation of data that was required;
- any LC_{50} (and 95% confidence limits) determined, and the statistical method used for calculation; and
- the results of toxicity tests with the reference toxicant(s) for the month of the test, together with the geometric mean value ($\pm$ 2 SD) for the same reference toxicant(s) as derived at the test facility in previous tests.

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Appendix A

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Appendix B

Conservation & Protection Regional and Headquarters Offices

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* Programs "BOOTSTRP" and "TOXSTAT" are available for copying onto a formatted 13-cm IBM-compatible floppy disk supplied by the user, by contacting the Laboratory Division at this address.

** A BASIC computer program for calculating LC_{50}s is available for copying onto a formatted 13-cm IBM-compatable floppy disk supplied by the user, by contacting the Aquatic Toxicity Laboratory at this address.

Appendix C

Use of Brine Shrimp for Feeding Fathead Minnows

Brine shrimp eggs can be purchased at aquarium supply stores and most pet stores. Also commercially available and recommended, are systems for hatching brine shrimp eggs, usually an elongated plastic bag and salts to provide water of 15 mg/kg salinity for these crustaceans. Instructions for using the apparatus and hatching the eggs are included with the particular device. Brine shrimp eggs can, however, be hatched in almost any conical container with air entering the narrow part at the bottom to keep the eggs in continual motion (Denny, 1987). The detailed advice and discussion on using brine shrimp provided by ASTM (1989) is commended.

Depending on the apparatus, the person making the preparation usually concentrates the hatched brine shrimp nauplii densely in their culture fluid before drawing them off. For feeding during the toxicity test, the shrimp should be rinsed in fresh water in order to avoid adding salt to the test solutions. The concentrate of brine shrimp nauplii in their culture medium should be rinsed in fresh water at 25°C in a separatory funnel, and allowed to settle for 2 min, during which time some of the unhatched eggs might conveniently float to the top. The settled concentrate of nauplii is then drawn into a small beaker or container with 20 µm screened bottom, and re-suspended for feeding to the fish with a repeater pipette or dropper (Neville, 1989). Usually, about 0.05 to 0.1 mL of the concentrate, i.e., 1 or 2 drops, will be sufficient for one feeding of a test vessel containing 10 larval fish. That feeding should contain about 700 to 1000 brine shrimp nauplii (Neville, 1989). A check should be made at the beginning of the procedural setup, to determine how many drops of shrimp concentrate are required to deliver those 700 to 1000 nauplii with the particular technique used in a laboratory (check by estimating numbers in an aliquot under microscope, using a haemocytometer or other appropriate device).

It is essential that all test vessels get the same amount of food, and positive techniques must be set up to accomplish that, such as mixing the suspension in the small beaker, and standard timing and positioning for refilling the dropper and delivering the nauplii. In addition, inspections should be made, particularly during early stages of testing in a laboratory, to make sure that there is a small excess of nauplii in the chambers throughout the daylight hours.

Two such feedings of the test vessels during each day should ensure near-maximum growth, if one is done early in the morning. Two feedings have been shown to achieve better growth of larvae than one feeding, although the difference is less noticeable if the single feeding is a heavy one (Silberhorn, 1989). Three feedings do not result in appreciably better growth than two feedings. Growth increases with more nauplii per day up to a plateau in the region of about 2000 nauplii per day.

For aquaria containing large numbers of fathead minnow larvae, proportionally more concentrate of brine shrimp nauplii would be required. For delivering shrimp to aquaria with a continuous flow of new water, it is

not necessary to rinse the shrimp in fresh water before delivery.

Because the larval minnows are very sensitive, and because the brine shrimp are their only food during the test, any contaminants in the food could be a distinct problem since they might cause combined action with toxicants being tested, and bias the results. Therefore, an effort should be made to use brine shrimp eggs which are known (by measurement) to contain low amounts of contaminants, especially persistent organochlorine compounds. U.S. EPA (1989) recommends chemical analysis of each new batch of brine shrimp eggs, with a maximum limit for use of 0.15 μg/g wet weight, of total organic chlorine. U.S. EPA also recommends brine shrimp eggs originating in Brazil or Columbia because of their record of low levels of contaminants, and gives a U.S. source for purchase. Because sources and suppliers will change for the brine shrimp commercially available in Canada, they are not specified here. The best indications of quality will come from measurement of contaminants in different supplies of eggs, success in hatching the shrimp and growing fathead minnows, and exchange of information on those subjects among laboratories.

The nutritive quality of brine shrimp might also vary with their origin. This factor is as difficult to assess on a continuing basis as the contamination question, but should be dealt with by keeping track of sources and success of rearing, and sharing information on measured nutritive values with other laboratories.

For adult or juvenile fathead minnows, foods other than frozen brine shrimp could be used. Other kinds of collected or cultured invertebrates or chopped meat, fresh or frozen, can be satisfactory or superior, but the frozen brine shrimp are convenient and of proven performance.

Appendix D

Logarithmic Series of Concentrations Suitable for Toxicity Tests*

Column (Number of concentrations between 100 and 10, or between 10 and 1)**						
1	2	3	4	5	6	7
100	100	100	100	100	100	100
32	46	56	63	68	72	75
10	22	32	40	46	52	56
3.2	10	18	25	32	37	42
1.0	4.6	10	16	22	27	32
	2.2	5.6	10	15	19	24
	1.0	3.2	6.3	10	14	18
		1.8	4.0	6.8	10	13
		1.0	2.5	4.6	7.2	10
			1.6	3.2	5.2	7.5
			1.0	2.2	3.7	5.6
				1.5	2.7	4.2
				1.0	1.9	3.2
					1.4	2.4
					1.0	1.8
						1.3
						1.0

* Modified from Rocchini *et al.* (1982).

** A series of five (or more) successive concentrations may be chosen from a column. Mid-points between concentrations in column (x) are found in column (2x + 1). The values listed can represent concentrations expressed as percentage by volume or weight, mg/L, or µg/L. As necessary, values may be multiplied or devided by any power of 10. Column 1 might be used if there was considerable uncertainty about the degree of toxicity. More widely spaced concentrations (differing by a factor <0.3) should not be used. For effluent testing, there is seldom much gain in precision by selecting concentrations from a column to the right of column 3; the finer gradations of columns 4 to 7 might occasionally be useful for testing chemicals that have an abrupt threshold of effect.

Appendix E

Analysis of Mortality to Estimate the Median Lethal Concentration

The 7-day growth and survival test with fathead minnow larvae is intended to be a sensitive and meaningful sublethal test, because early life-stages are usually among the most sensitive in a life-cycle test. Therefore, this assay usually focuses on determining the NOEC/LOEC (or the geometric average of those concentrations) and/or the ICp (see Section 4.5). However, mortality of larval fathead minnows is a relatively sensitive effect in the life cycle, and is sometimes the most sensitive effect that is documented, particularly in the seven-day larval exposure (Woltering, 1984; McKim, 1985; Suter *et al.*, 1987). Therefore, a point-estimate of the concentration causing lethality can be useful. The methods for estimating the LC_{50} are well-established and dependable, and some recommendations for making such an estimate are given here.

A larval LC_{50} obtained in the course of the present test would be much lower than the usual acute LC_{50}s for fathead minnows that are recorded in the literature, since they are obtained from tests with more tolerant juvenile fish.

To estimate an LC_{50}, data are combined for all replicates at each concentration, for a given exposure-time that would normally be seven days, the length of this test. If mortality is not ≥50% in at least one concentration, the LC_{50} cannot be estimated. If there is no mortality at a certain concentration, that information is used in fitting the probit line, being an effect of 0% mortality. However, if successive concentrations yield a series of 0% mortalities, only one such value should be used in estimating the LC_{50}, and that should be the highest concentration of the series, i.e., the zero-effect that is "closest to the middle" of the distribution of data. Similarly, if there were a series of successive complete mortalities at the high concentrations in the test, only one value of 100% effect would be used, again the one "closest to the middle", i.e., the 100% effect at the lowest of these concentrations. Use of only one 0% and one 100% effect applies to analyzing the data by computer program or to hand plotting on a graph (see below). Using additional values of 0% and/or 100% might distort the estimate of LC_{50}.

Various computer programs for calculating LC_{50} and confidence limits are suitable for use. Stephan (1977) developed a program for estimating the LC_{50} which uses probit, moving average, and binomial methods, and adapted it for the IBM-compatible personal computer. This program in the BASIC language is recommended, and is available for copying onto a user-supplied floppy diskette through courtesy of Dr. Charles E. Stephan (U.S. EPA, Duluth, MN), from Environment Canada (Appendix B). An efficient micro-computer program for probit analysis is also available from J.J. Hubert (1987), and other satisfactory computer and manual methods (APHA *et al.*, 1989, U.S. EPA, 1989) may be used. Programs using the Trimmed Spearman-Karber method (Hamilton *et al.*, 1977) are available for personal computers but are not recommended here because

divergent results might be obtained by operators who are unfamiliar with the implications of trimming off ends of the dose-response data.

The recommended program of C.E. Stephan provides estimates of LC_{50} and confidence limits by each of its three methods, if there are at least two partial mortalities in the set of data. For smooth or regular sets of data, the three estimates will likely be similar (see following text), and values from the probit analysis should be taken as the preferred ones and reported. The binomial estimate might differ somewhat from the others. If the results do not include two partial mortalities, only the binomial method functions, and it can be used to provide a best estimate of the LC_{50} with conservative (wide) confidence limits.

Any computer-derived LC_{50} should be checked by examining a plot on logarithmic-probability scales, of % mortalities at a fixed observation-time (e.g., seven days) for the various test concentrations (APHA *et al.*, 1989; see example in Figure E.1). Any major disparity between the estimated LC_{50} derived from this plot and the computer-derived LC_{50} must be resolved.

In the hypothetical example shown in Figure E.1, ten fish were tested at each of five concentrations (1.8, 3.2, 5.6, 10, and 18 mg/L), and a control (with no control mortality). Mortalities in the test concentrations, of 0, 2, 4, 9, and 10 fish, were plotted and a line fitted by eye. The concentration expected to be lethal to half the fish was read by following across from 50% (broken line) to the intersection with the eye-fitted line, then down to the horizontal axis, where an estimated LC_{50} of 5.6 mg/L was read off.

In fitting a line such as that in Figure E.1, relatively more emphasis should be assigned to points that are near 50% mortality. Logarithmic-probability paper ("log-probit", as in Figure E.1) can be purchased in good technical bookstores, or ordered through them.

Computer programs gave very similar estimates to the graphic one, for the regular set of data in Figure E.1. The LC_{50}s (and 95% confidence limits) were as follows:

Probit analysis of Hubert (1987):		5.56	(4.28 to 7.21)
Stephan (1977)	probit analysis:	5.58	(4.24 to 7.37)
	moving average:	5.58	(4.24 to 7.33)
	binomial:	6.22	(1.8 to 10)
Spearman-Kärber method:	0% trim:	5.64	(4.38 to 7.26)
(Hamilton *et al.*, 1977)	10% trim:	5.73	(4.34 to 7.58)
	20% trim:	5.95	(4.34 to 9.80)

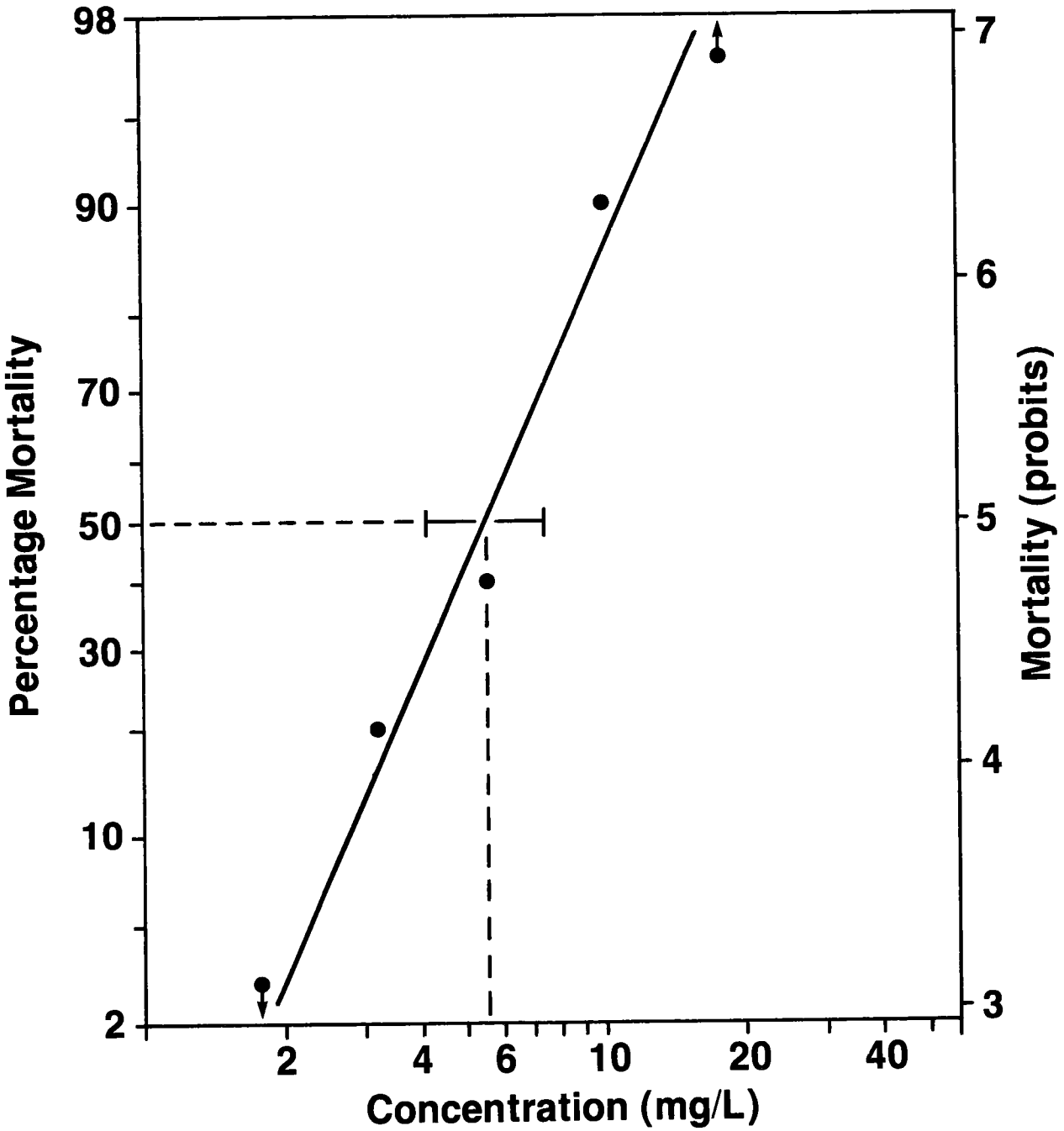

Figure E.1 **Estimating a Median Lethal Concentration by Plotting Mortalities on Logarithmic-probability Paper**

The binomial method did not estimate confidence limits, but selected two concentrations from the test as outer limits of a range within which the true confidence limits would lie.

It is also possible, if desired, to estimate the "time to 50% mortality" (LT_{50}) in a given concentration. A graph similar to Figure E.1 can be plotted using logarithm of time as the horizontal axis. Individual times to death of organisms would not be available for use, since tests would not be inspected continuously. The cumulative % mortality at successive inspections (normally, daily) is quite satisfactory for plotting, and an eye-fitted line leads to estimates of confidence limits following the steps listed in Litchfield (1949). Data permitting, such LT_{50}s could be estimated from successive records of mortality at 24-h intervals.

Observed mortality must be greater than 50% in order to estimate an LT_{50}.

Neither an LT_{50} nor the percentage mortality at short exposure-times is a dependable method of judging ultimate toxicity, thus comparisons based on those endpoints give only semi-quantitative guidance. However, it might sometimes be useful to document whether the material being tested is rapidly or slowly lethal; for example, it might give guidance on a question of regulatory allowances for short-term excursions in concentration above a long-term permitted limit. In theory, deriving LT_{50}s instead of an LC_{50} can allow more complete utilization of information from the test, and a time-concentration curve of lethality might provide useful insight for investigating mechanisms of effect (Sprague, 1969; Suter *et al.*, 1987).